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| (54) Title: ATP BINDING CASSETTE GENES AND PROTEINS FOR DIAGNOSIS AND TREATMENT OF LIPID DISORDERS AND INFLAMMATORY DISEASES | | |
| (54) Titre: GENES ET PROTEINES DE CASSETTE DE LIAISON AVEC ATP, DESTINES AU DIAGNOSTIC ET AU TRAITEMENT DE DESORDRES LIPIDIQUES ET MALADIES INFLAMMATOIRES | | |
| (57) Abstract | | |
| <p>Modulation of the activity of transmembrane proteins belonging to the ATP binding cassette (ABC) transporter protein family which are etiologically involved in cholesterol driven atherogenic processes and inflammatory diseases like psoriasis, lupus erythematoses and others provides therapeutic means to treat such diseases. Furthermore, detection of herein identified ABC transporter proteins of their respective biochemical activities involved in such atherogenic and inflammatory processes provides diagnostic means for clinical application of diagnosis and monitoring of dyslipidemias, atherosclerosis or inflammatory diseases like psoriasis and lupus erythematoses.</p> | | |
| (57) Abrégé | | |
| <p>Selon l'invention, la modulation de l'activité de protéines transmembranaires qui appartiennent à la famille de protéines de transport (ABC) de cassette de liaison avec ATP et sont impliquées de manière étiologique dans des processus athérogènes provoqués par le cholestérol et dans des maladies inflammatoires comme le psoriasis, le lupus érythémateux et autres, constitue un moyen thérapeutique de traiter de telles maladies. En outre, la détection des protéines de transport (ABC) ici identifiées et de leurs activités biochimiques respectives, impliquées dans de tels processus athérogènes et inflammatoires, constitue un moyen de diagnostic destiné à l'application clinique de diagnostic et de surveillance des dyslipidémies, de l'athérosclérose ou de maladies inflammatoires telles que le psoriasis ou le lupus érythémateux.</p> | | |

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Description

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ATP binding cassette genes and proteins for diagnosis and treatment of lipid disorders and inflammatory diseases

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Background of the invention

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Reverse cholesterol transport mediated by HDL provides a "protective" mechanism for cell membrane integrity and foam cell formation and cellular cholesterol is taken up by circulating HDL or its precursor molecules. The precise mechanism of reverse cholesterol transport however is currently not fully understood and the mechanism of cellular cholesterol efflux and transfer from the cell surface to an acceptor-particle, such as HDL, is yet unclear. Certain candidate gene products have been postulated playing a role in the process of reverse cholesterol transport [1]. Apolipoproteins (e.g. ApoA-I, ApoA-IV), lipid transfer proteins (e.g. CETP, PLTP) and enzymes (e.g. LCAT, LPL, HL) are essential to exchange cholesterol and phospholipids in lipoprotein-lipoprotein and lipoprotein-cell interactions. Different plasma membrane receptors, such as SR-BI [2; 3], HB1/2 [4], and GPI-linked proteins (e.g. 120 kDa and 80 kDa) [5] as well as the sphingolipid rich microdomains (Caveolae, Rafts) of the plasma membrane have been implicated being involved in the process of reverse cholesterol transport and the exchange of phospholipids. How these membrane-microdomains are organized is in the current focus of interest for the identification of therapeutic targets. In recent studies SR-BI function as receptor for uptake of HDL into the liver and steroidogenic tissues could be demonstrated and the effectivity of this process is highly dependent on the phospholipid environment [2].

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Cholesterol and phospholipid homeostasis in monocytes/macrophages and other cells involved in the atherosclerotic process is a critical determinant in atherosclerotic vessel disease. The phagocytic function of macrophages in host defense, tissue remodelling, uptake and lysosomal degradation of atherogenic lipoproteins and membrane fragments or other lipid containing particles has to be balanced by effective release mechanisms to avoid foam cell formation. HDL mediated reverse

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cholesterol transport, supported by endogenous ApoE and CETP synthesis and secretion provides an effective mechanism to release excessive cholesterol from macrophages and other vascular cells.

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- 5 Alternatively, reduced cholesterol and triglyceride/fatty acid absorption by intestinal mucosa cells as well as increased lipid secretion from hepatocytes into the bile will lower plasma lipids and the concentration of atherosclerotic lipoproteins.

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Summary of the invention

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New cholesterol responsive genes were identified with differential display method in human monocytes from peripheral blood that were subjected to macrophage differentiation and cholesterol loading with acetylated LDL and subsequent deloading with HDL₃.

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In an initial screen ABCG1 (ABC8), a member of the rapidly growing family of ABC (ATP-Binding Cassette) transport systems, that couple the energy of ATP hydrolysis to the translocation of solutes across biological membranes, was identified as a cholesterol sensitive switch. ABCG1 is upregulated by M-CSF dependent phagocytic differentiation but expression is massively induced by cholesterol loading and almost completely set back to differentiation dependent levels by HDL₃.

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20 In a more detailed analysis 37 already characterised ABC members and 8 Fragment - sequences (Table 2) were analysed in monocyte/macrophage cells by RT-PCR (linear range) for differentiation dependent changes and cholesterol sensitivity.

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Among the 45 tested ABC-transporter genes 18 of the characterized ABC transporters and 2 of the Fragment -sequence based ABC-transporters are cholesterol sensitive (Example 4).

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The cholesterol sensitive ABC-transporter are named according to the new ABC-

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nomenclature and listed in Table 3 with the new and the old designations, respectively.

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The most sensitive gene was ABCG1. ABCG1 is the human homologue of the drosophila white gene. Sequencing of the promoter of ABCG1 (Example 7) shows important transcription factor binding sites relevant for phagocytic differentiation and lipid sensitivity.

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Antisense treatment of macrophages during cholesterol loading and HDL₃-mediated deloading clearly identified ABCG1 as a cholesterol transporter and the efflux of choline-containing phospholipids (phosphatidylcholine, sphingomyelin) was also modulated. Northern- and Western-blot analysis provided further support that inhibition of cholesterol transport is associated with lower ABCG1 mRNA expression and ABCG1 protein levels (Example 5).

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Considerable evidence was derived from energy transfer experiments (Example 3) that ABCG1 in the cell membrane is in a regulated functional cooperation (e.g. cell differentiation, activation, cholesterol loading and deloading) with other membrane receptors that have either transport- (e.g. LRP-LDL receptor related protein) or signalling- and adhesion-function (e.g. integrins, integrin associated proteins) which is also supported by sequence homology of extracellular domains as well as other parts of the ABCG1 sequence. For example the protein sequence of the region of the third extracellular loop of ABCG1, i.e. aminoacid residues 580 through 644, shares homology with fibronectin (aa 317-327), integrin β 5 (aa 538-547), RAP (aa 119-127), LRP (aa 2874-2894), apoB-100 precursor (aa 4328-4369), glutathion-S-transferase (aa 54-78) and glucose transporter (aa 371-380). Sequence comparison of all cholesterol sensitive transporters indicates this as a general principle of ABC transporter function and regulation.

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Among the other cholesterol sensitive genes ABCA1 (ABC1) was further characterized. ABCA1 was identified in the mouse as an IL-1 β transporter

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involved also in apoptotic cell processing. We show here, by RT-PCR (Table 2) and confirmation by Northern analysis, based on the newly detected human ABCA1 cDNA sequence (Example 6), that ABCA1 follows the same regulation as ABCG1.

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5 Moreover, the ABCA1-knockout mice (ABCA1^{-/-}) show massively reduced levels of serum lipids and lipoproteins. The expression of ABCA1 in mucosa cells of the small intestine and the altered lipoprotein metabolism in ABCA1^{-/-} mice allows the conclusion that ABCA1 plays a major role in intestinal absorption and translocation of lipids into the lymph-system

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Analysis of genetic defects that affect macrophage cholesterol homeostasis identified dysregulated ABCA1 as a gene locus involved in the HDL-deficiency syndrome (Tangier-Disease). This disease is associated with hypertriglyceridemia and splenomegaly.

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15 Another as yet not described HDL-deficiency syndrome associated with early onset of coronary heart disease and psoriasis showed a dysregulation of the chromosome 17 associated ABC-sequences (ABCC4 (MRP3); ABCC3 (MRP3); ABCA5 (Fragment 90625); ABCA6 (Fragment 155051) :17q21-24). This points to an 20 association with the predicted gene locus for psoriasis at chromosome 17.

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A recently sequenced human ABC-transporter (ABCA8, Example 9) shows high homology to ABCA1 and also belongs to the group of cholesterol sensitive ABC-transporter.

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25 ABCC5 (MRP5, sMRP) is a member of the MRP-subfamily among which ABCC2 (MRP2, cMOAT) was characterized as the hepatocyte canalicular membrane transporter that is involved in bilirubin glucuronide secretion [9] and identified as the 30 gene locus for Dubin-Johnson Syndrome [10] a disorder associated with mild chronic conjugated hyperbilirubinemia.

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Furthermore, the identification of ABCA1 as a transporter for IL-1 β identifies this gene as a candidate gene for treatment of inflammatory diseases including rheumatoid arthritis and septic shock. The cytokine IL-1 β is a broadly acting proinflammatory mediator that has been implicated in the pathogenesis of these diseases.

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Moreover, we could demonstrate, that glyburide as an inhibitor of IL-1 β secretion inhibits not only Caspase I mediated processing of pro-IL-1 β and release of mature IL-1 β but simultaneously inhibits ceramide formation from sphingomyelin mediated by neutral sphingomyelinase and thereby releases human fibroblasts from G₂-phase cell cycle arrest. These data provide a further mechanism indicative for a function of ABCA1 in signalling and cellular lipid metabolism.

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Autoimmune disorders that are associated with the antiphospholipid syndrome (e.g. lupus erythematoses) can be related to dysregulation of B-cell and T-cell function, aberrant antigen processing, or aberrations in the asymmetric distribution of membrane phospholipids. ABC-transporters are, besides their transport function, candidate genes for phospholipid translocases, floppases and scramblases that regulate phospholipid asymmetry (outer leaflet: PC+SPM; inner leaflet: PS+PE) of biological membranes [11]. There is considerable evidence for a dysregulation of the analysed ABC-transporters in patient cells. We conclude that these ABC-cassettes are also candidate genes for a genetic basis of antiphospholipid syndromes such as in Lupus erythematoses.

In summary, the ABC genes ABCG1, ABCA1 and the other cholesterol-sensitive ABC genes as specified herein, can be used for diagnostic and therapeutic applications as well as for biochemical or cell-based assays to screen for pharmacologically active compounds which can be used for treatment of lipid disorders, atherosclerosis or other inflammatory diseases. Thus it is an objective of the present invention to provide assays to screen for pharmacologically active compounds which can be used for treatment of lipid disorders, atherosclerosis or

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other inflammatory diseases. Further the invention provides tools to identify modulators of these genes and gene products. These modulators can be used for the treatment of lipid disorders, atherosclerosis or other inflammatory diseases or for the preparation of medicaments for treatment of lipid disorders, atherosclerosis or other inflammatory diseases. The medicaments comprise besides the modulator acceptable and usefull pharmaceutical carriers.

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Abbreviations

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|----|----------|---|
| | aa | Amino acid |
| | ABC | ATP-binding cassette |
| 15 | ABCA# | ATP-binding cassette, sub-family A (ABC1), member # |
| | ABCB# | ATP-binding cassette, sub-family B (MDR/TAP), member # |
| | ABCC# | ATP-binding cassette, sub-family C (CFTR/MRP), member # |
| | ABCD# | ATP-binding cassette, sub-family D (ALD), member # |
| 20 | ABCE# | ATP-binding cassette, sub-family E (OABP), member # |
| | ABCF# | ATP-binding cassette, sub-family F (GCN20), member # |
| | ABCG# | ATP-binding cassette, sub-family G (WHITE), member # |
| 25 | ABCR | Homo sapiens rim ABC transporter |
| | AcLDL | Acetylated LDL |
| | ADP1 | ATP-dependent permease |
| | ALDP | Adrenoleukodystrophy protein |
| 30 | ALDR | Adrenoleukodystrophy related protein |
| | ApoA | Apolipoprotein A |
| | ApoE | Apolipoprotein E |
| 35 | ARA | Anthracycline resistance associated protein |
| | AS | Antisense |
| | ATP | Adenosine triphosphate |
| | CETP | Cholesteryl ester transfer protein |
| 40 | CFTR | Cystic fibrosis transmembrane conductance regulator |
| | CGT | ceramide glucosyl transferase |
| | CH | Cholesterol |
| 45 | cMOAT | Canalicular multispecific organic anion transporter |
| | dsRNA | Double stranded RNA |
| | Fragment | Gen Fragment |
| 50 | FABP | plasma membrane fatty acid binding protein |

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| 5 | FACS | Fluorescence activated cell sorter |
| 10 | FATP | intracellular fatty acid binding protein |
| | FCS | foetal calf serum |
| | FFA | free fatty acids |
| 15 | GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| | GCN20 | protein kinase that phosphorylates the alpha-subunit of translation initiation factor 2 |
| | GPI | Glycosylphosphatidylinositol |
| 20 | HaCaT | keratinocytic cell line |
| | HDL | High density lipoprotein |
| | HL | Hepatic lipase |
| | IllyB | haemolysin translocator protein B |
| 25 | HMT1 | yeast heavy metal tolerance protein |
| | HPTLC | High performance thin layer chromatography |
| | IL | Interleukin |
| 30 | LCAT | Lecithin:cholesterol acyltransferase |
| | LDL | Low density lipoprotein |
| | LPL | Lipoprotein lipase |
| | LRP | LDL receptor related protein |
| 35 | MDR | Multidrug resistance |
| | MRP | Multidrug resistance-associated protein |
| | PC | Phosphatidylcholine |
| 40 | PE | Phosphatidylethanolamin |
| | PL | Phospholipid |
| | PLTP | Phospholipid transferprotein |
| | PMP | peroxisomal membrane protein |
| 45 | PS | Phosphatidylserine |
| | RNA | Ribonucleic acid |
| | RT-PCR | Reverse transcription – polymerase chain reaction |
| 50 | SDS | Sodium dodecyl sulfate |

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| | SL | Sphingolpid |
| | sMRP | Small form of MRP |
| 10 | SPM | Sphingomyelin |
| | SR-BI | Scavenger receptor BI |
| | SUR | Sulfonylurea receptor |
| 15 | TAP | Antigen peptide transporter |
| | TG | Triglycerides |
| | TSAP | TNF-alpha stimulated ABC protein |
| | UTR | untranslated region |

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Description of the Figures

10 Figures 1 to 5 are showing nucleotide and protein sequences described in this application. The sequences are repeated in the sequence listing.

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Description of Tables:

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Table 1:

20 Levels of RNA transcripts of ABCG1 (ABC8), ABCA1 (ABC1) and ABCA8 in human tissues were determined by Northern blot analysis of a multiple tissue dot-blot (Human RNA MasterBlot, Clontech Laboratories, Inc., CA, USA). The relative amount of expression is indicated by different numbers of filled circles.

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Table 2:

15 The expression pattern of ABC-transporters in monocytes, monocyte derived macrophages (3 days cultivated monocytes in serum free Macrophage-SFM medium containing 50 ng/ml M-CSF), AcLDL incubated monocytes (3 days with 100 µg/ml) followed by HDL₃ (100 µg/ml) incubated monocytes is shown. Expressed genes are tested for cholesterol sensitivity by semiquantitative PCR.
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35 For known ABC-Transporter the chromosomal location and the transported molecules are also presented.

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Table 3:

40 Disorders, that are associated with ABC-transporters are shown. The chromosomal location is indicated and the relevant accession number in OMIN (Online Mendelian Inheritance in Man).

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Table 4:

30 Expression of ABC-Transporters in HaCaT keratinocytic cells during differentiation

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Table 1

| <i>Tissue</i> | ABCG1 (ABC8) | ABCA1 (ABC1) |
|-----------------------|-----------------|-----------------|
| Adrenal gland | ••••• | ••• |
| Thymus | ••••• | •• |
| Lung | ••••• | ••• |
| Heart | ••• | •• |
| Skeletal | •• | • |
| Brain | ••• | •• |
| Spleen | ••••• | •• |
| Lymphnode | ••• | • |
| Pancreas | • | • |
| Placenta | ••••• | ••••• |
| Colon | •• | • |
| Small intestine | •• | •••• |
| Prostate | •• | • |
| Testis | • | • |
| Ovary | •• | • |
| Uterus | • | •• |
| Mammary gland | •• | • |
| Thyroid gland | •• | •• |
| Kidney | •• | • |
| Liver | ••• | ••• |
| Bone marrow | • | • |
| Peripheral leukocytes | • | • |
| <i>Fetal tissue</i> | | |
| Fetal brain | • | •• |
| Fetal liver | • | •••• |
| Fetal spleen | •• | ••• |
| Fetal thymus | •• | •• |
| Fetal lung | •• | ••• |

Table 2: Cholesterol dependent gene regulation of human ABC transporters

| | Gene | chromosomal localization | peripheral blood monocytes | 3 days old M-CSF Mf | cholesterol loading (acLDL) | cholesterol deloading (HDL3) | transported molecules |
|----|-------------------------|--------------------------|----------------------------|---------------------|-----------------------------|------------------------------|------------------------------|
| 10 | ABCG1 (ABC8) | 21q22.3 | + | ↑ | ↑↑ | ↓↓ | cholesterol / choline PL |
| 15 | ABCA1 (ABC1) | 9q22-31 | + | ↑ | ↑↑ | ↓↓ | cholesterol / IL-1 \square |
| 20 | ABCC5 (MRP5) | 3q25-27 | + | ↑ | ↑↑ | ↓ | |
| 25 | ABCD1 (ALDP, ALD) | Xq28 | + | ↑ | ↑ | ↓ | very long chain fatty acids |
| 30 | ABCA5 (est90625) | 17q21-25 | + | ↑ | ↑ | ↓ | |
| 35 | ABCB11 (BSEP, SPGP) | 2q24 | + | ↑ | ↑↑ | ↓ | bile acids |
| 40 | ABCA8 (ABC-new) | | + | + | ↑ | ↓ | |
| 45 | ABCC2 (MRP2) | 10q23-24 | + | + | ↑ | ↓ | bilirubin glucuronide |
| 50 | ABCB6 (est45597) | 2q33-36 | + | + | ↑ | ↓ | |
| | ABCC1 (MRP1) | 16p13.12 | + | ↓ | ↑ | ↓ | eicosanoids |
| | ABCA3 (ABC3) | 16p13.3 | + | ↑ | ↑ | nr | |
| | est1133530 | | + | ↑ | ↑ | nr | |
| | ABCB4 (MDR3) | 7q21 | + | ↑ | ↓ | ↑ | phosphatidylcholine |
| | ABCG2 (est157481, ABCP) | 4q22-23 | + | ↑ | ↓ | ↑ | |
| | ABCC4 (MRP4) | 13q31 | + | ↑ | ↓ | ↑ | |
| | ABCB9 (est122234) | 12q24 | + | ↑ | ↓ | ↑ | |
| | ABCD2 (ALDR) | 12q11 | + | ↓ | ↓ | ↑ | very long chain fatty acids |
| | ABCB1 (MDR1) | 7q21 | + | + | ↓ | ↑ | phospholipids, amphiphiles |
| | ABCA6 (est155051) | 17q21 | + | ↑ | ↓ | nr | |
| | est1640918 | | + | ↑ | ↓ | nr | |
| | ABCD4 (P70R) | 14q24.3 | + | ↑ | nr | nr | |
| | ABCA2 (ABC2) | 9q34 | + | ↑ | nr | nr | |
| | ABCF2 (est133090) | 7q35-36 | + | ↑ | nr | nr | |
| | ABCB7 (ABC7) | Xq13.1-3 | + | ↑ | nr | nr | iron |
| | ABCF1 (ABC50, TSAP) | 6p21.33 | + | ↑ | nr | nr | |
| | ABCC6 (MRP6) | 16p13.11 | + | ↓ | nr | nr | |
| | ABCB5 (est422562) | 7p14 | + | ↓ | nr | nr | |
| | ABCC3 (MRP3) | 17q11-21 | + | nr | nr | nr | |
| | ABC \wedge 4 (ABCR) | 1p22 | + | nr | nr | nr | retinoids, lipofuscin |
| | ABCB2 (TAP1) | 6p21.3 | + | nr | nr | nr | peptides |
| | ABCB3 (TAP2) | 6p21.3 | + | nr | nr | nr | peptides |

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| Gene | chromosomal localization | peripheral blood monocytes | 3 days old M-CSF MO | cholesterol loading (acLDL) | cholesterol unloading (HDL3) | transported molecules |
|--------------------|--------------------------|----------------------------|---------------------|-----------------------------|------------------------------|-----------------------|
| ABCF3 (est201864) | 3q25.1-2 | + | nr | nr | nr | |
| ABCB8 (est328128) | 7q35-36 | + | ↑ | nr | nr | |
| ABCE1 (OABP) | 4q31 | + | ↑ | nr | nr | |
| ABCB10 (est20237) | 1q32 | + | ↑ | nr | nr | |
| est698739 | | + | ↑ | nr | nr | |
| ABCC10 (est182763) | 6p21 | + | nr | nr | nr | |
| ABCC7 (CFTR) | 7q31 | ∅ | ∅ | ∅ | ∅ | ions |
| ABCC8 (SUR-1) | 11p15.1 | ∅ | ∅ | ∅ | ∅ | |
| ABCD3 (PMP70) | 1p21-22 | ∅ | ∅ | ∅ | ∅ | |
| Huwhite2 | | ∅ | ∅ | ∅ | ∅ | |
| est1125168 | | ∅ | ∅ | ∅ | ∅ | |
| est1203215 | | ∅ | ∅ | ∅ | ∅ | |
| est168043 | | ∅ | ∅ | ∅ | ∅ | |
| est990006 | | ∅ | ∅ | ∅ | ∅ | |

+ = expressed

∅ = not expressed

nr = not regulated

↑ = upregulated

↓ = downregulated

half (hs) or full size (fs) transporter as deduced from the mRNA size

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Table 3

| <i>Disorders</i> | <i>Genomic location</i> | <i>Associated gene</i> | <i>OMIM-acc.nr.</i> |
|---|-------------------------|----------------------------|---------------------|
| <i>Metabolic disorders:</i> | | | |
| Cystic fibrosis | 7q31.3 | ABCC7 (CFTR) | 219700 |
| Dubin Johnson syndrome (mild chronic conjugated hyperbilirubinemia) | 10q24 | ABCC2 (CMOAT) | 237500 |
| Progressive familial intrahepatic cholestasis type III (PIFC3) | 7q21.1 | ABCB4 (MDR3) | 602347 |
| <i>Byler disease (PFIC2)</i> | <i>2q24</i> | <i>ABCB11 (BSEP, sPGP)</i> | <i>601847</i> |
| Familial persistent hyperinsulinemic hypoglycemia | 11p15.1 | ABCC8 (SUR-1) | 601820 |
| IDDM | 6p21.3 | ABCB2 (TAP1)/ABCB3 (TAP2) | 222100 |
| <i>Neuronal disorders:</i> | | | |
| Adrenoleukodystrophy | 12q11 | ABCD2 (ALDR) | 300100 |
| Zellweger's syndrome | 1p22-21 | ABCD3 (PMP70) | 214100 |
| Multiple Sclerosis | 6p21.3 | ABCB2 (TAP1)/ABCB3 (TAP2) | 126200 |
| X-linked Sideroblastic anemia with spinocerebellar ataxia | Xq13.1-3 | ABCB7 (ABC7) | 301310 |
| Menkes disease (altered homeostasis of metals) | Xq13 | ABCB7 (ABC7) | 309400 |
| <i>Immune/Hemostatic disorders:</i> | | | |
| Herpes simplex virus infection [12] | 6p21.3 | ABCB2 (TAP1)/ABCB3 (TAP2) | |
| Behcet's syndrome | 6p21.3 | ABCB2 (TAP1)/ABCB3 (TAP2) | 109650 |
| Bare lymphocyte syndrome type I | 6p21.3 | ABCB2 (TAP1)/ABCB3 (TAP2) | 209920 |
| Scott syndrome | 7q21.1 | ABCB1 (MDR1) | 262890 |
| <i>Retinal dystrophies:</i> | | | |
| Fundus flavimaculatus with macular dystrophy | 1p13-21 | ABCA4 (ABCR) | 601691 |
| Juvenile Stargardt disease | 1p13-21 | ABCA4 (ABCR) | 248200 |
| Age-related macular degeneration | 1p13-21 | ABCA4 (ABCR) | 153800 |
| Cone-rod dystrophy | 1p13-21 | ABCA4 (ABCR) | 600110 |
| Retinitis pigmentosa | 1p13-21 | ABCA4 (ABCR) | 601718 |

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| <i>Diseases with evidence for involvement of ATPcassettes/translocases and floppases(80)</i> | | | | <i>Assumed gene</i> |
|--|----------------------|--|----------------------------|---------------------|
| BRIC (Benign recurrent intrahepatic obstructive jaundice) | 18 | Assumed | 243300 | |
| Psoriasis | 17q11-12 17q21-24 | ABCAS (Fragment 90625) ABCC3 (MRP3) | 602723 177900 601454 | |
| Lupus erythematoses – Antiphospholipid Syndrome | | Translocase Flippase | 152700 | |
| PFIC(Prog. Fatal familial intrahepatic choestasis) PFIC1 | 18q21-22 | ATP Transporters | 211600 | |
| <i>Neurological disorders mapped to gene locus of ABCG1 (ABC8)</i> | | | | |
| Autosomal bipolar affective disorder | 21q22.3 | ABCG1 (ABC8) | 125480 | |
| Autosomal recessive non-syndromic deafness | 21q22.3 | ABCG1 (ABC8) | 601072 | |
| Down Syndrome (ABC-8 may be a candidate for the Brushfield spots – mottled, marble or speckled irides frequently seen in Down-Syndrome) | 21q22.3 | ABCG1 (ABC8) | 190685 | |
| Linkage to phosphofructokinase (liver type) | 21q22 | | 171860 | |
| HDL-deficiency syndromes, Gen responsible for Tangier Disease | 9q31 | ABCA1 (ABC1) | 205400 | |

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Table 4: Expression of ABC-Transporters in HaCaT keratinocytic cells during differentiation

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| <i>Gene</i> | chrom. localisation | initial expression | differentiation dependent expression | known or putative molecules transported |
|---------------------|---------------------|--------------------|--------------------------------------|---|
| ABCG1 (ABCR) | 21 q22.3 | +++++ | ↑ | cholesterol choline-PL |
| ABCC3 (MRP3) | 17 q11-q12 | +++++ | ↑ | |
| ABCA8 | 19 p13 | +++++ | ↑ | |
| ABCC1 (MRP1) | 16 p13 | +++++ | ↗ ↘ (max. day 2) | PGA _i , LTC _i DNP-SG |
| ABCD4 (PMP69, P70R) | 14 q24 | ++++ | ↗ ↘ (max. day 2,4) | |
| ABCC2 (MRP2) | 10 q24 | +++ | ↗ ↘ (max. day 2) | bilirubin glucuronide |
| ABCA3 (ABC3) | 16 p13 | + | ↗ ↘ (max. day 4,6) | |
| ABCA5 (ABCR) | 1 p21 | + | ↗ ↘ (max. day 4) | retinoid, lipofuscin |
| ABCA1 (ABC1) | 9 q22-q31 | + | ↗ ↘ (max. day 6) | |
| ABCC6 (MRP6) | 16 p13.11 | + | ↗ ↘ (max. day 4) | |
| ABCC4 (MRP4) | 13 q31 | ++++ | ↗ ↘ (max. day 2,4) | |
| ABCA2 | 9 q34 | ++++ | ↗ ↘ (max. day 6) | |
| ABCC5 (MRP5, SMRP) | 3 q27 | +++++ | ↗ ↘ (max. day 2,4) | |

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|----|---------------------------|-------------|-------|--------------------|----------|
| | ABCB6 (est45597) | 2 | +++++ | ↗ ↘ (max. day 2,4) | |
| 10 | ABCB7 (ABC7) | X q13.1-3 | +++++ | ↗ ↘ (max. day 4) | irons |
| 15 | TAP1 (ABCB1) | 6 p21.3 | +++++ | ↗ ↘ (max. day 4,6) | peptides |
| | TAP2 (ABCB2) | 6 p21.3 | +++++ | ↗ ↘ (max. day 2,4) | |
| 20 | ABCB8 (est328128) | 7 q35-36 | +++++ | ↗ ↘ (max day 2) | |
| 25 | EST640918 | 17 q24 | + | ↗ ↘ (max day 4) | |
| | ABCC7 (CFTR) | 7 q31 | +++ | ↗ ↘ (max day 4) | |
| 30 | ABCB10 (est20237) | 1 q32 | +++ | ↗ ↘ (max. day 2) | |
| | ABCF1 (TSAP) | 6 p21.33 | +++++ | ↓ | |
| 35 | ABCC10 (est182763) | 1 q32 | +++++ | ↓ | |
| | ABCE1 (OABP) | 4 q31 | +++++ | ↓ | |
| 40 | EST698739 | 17 q24 | +++++ | ↓ | |
| | ABCF2 (est133090) | 7 q35-q36 | +++++ | ↓ | |
| 45 | ALD (ABCD1,ALDP) | X q28 | ++++ | ↓ | VLCFA |
| | ABCA5 (est90625) | 17 q21-q24 | +++ | ↓ | |
| 50 | ABCB5 (est422562) | 7 p14 | ++++ | ↓ | |
| | ABCB9 (est122234) | 12 q24-qter | ++ | ↓ | |
| | ABCD2 (ALDR) | 12 q11 | + | ↓ | VLCFA |
| | ABCF3 (est201864) | 3 q25.1-2 | +++++ | ↓ | |
| | ABCG2 (ABC15,ABCP) | 4 q22-q23 | ++++ | ↓ | |
| | EST1133530 | 4 p16pter | +++++ | ↓ | |

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|--|---------------------------|----------|---------------|----------------|---------------------------|
| | Huwhite | 11 q23 | ++++ | ↓ | |
| | ABCA6 (est155051) | 17 q21 | ++ | ↓ | |
| | BSEP (ABCB11,sPGP) | 2 q24 | + | ↓↑ (max day 6) | |
| | ABCB4 (MDR3) | 7 q21 | not expressed | | phosphatidyl-choline |
| | ABCD3 (PMP70) | 1 p22 | not expressed | | |
| | ABCB1 (MDR1) | 7 q21 | not expressed | | phospholipids amphiphiles |
| | EST168043 | 2 p15-16 | not expressed | | |
| | EST990006 | 17 q24 | not expressed | | |
| | ABCC8 (SUR1) | 11 p15.1 | not expressed | | |

+: relative expression n.d.: not determined

↑: upregulated ↓: downregulated ▲: biphasic expression

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Description of specific embodiments

10 Candidate gene identification during cholesterol loading and deloading of
human monocyte derived macrophages

15 In order to discover genes that are involved in the cholesterol loading and/or
deloading in vitro assays were set up. Particularly, gene expression in human blood
derived monocytes and macrophages elicited by cholesterol and its physiological
transport formulation, i.e. various low density lipoprotein (LDL) particle species like
20 AcLDL, was studied.

25 Elutriated human monocytes were cultivated in M-CSF containing but serum free
macrophage medium supplemented with AcLDL (100 µg protein/ml medium) for
three days, followed by cholesterol depletion replacing AcLDL by HDL₃ (100 µg
15 protein/ml medium) for twelve hours. Differential display screening for new
candidate genes, regulated by cholesterol loading/deloading, was performed
30 (Example 1).

Identification of a new cholesterol sensitive gene

20 ABCG1 (ABC8) was discovered as a novel cholesterol sensitive gene. ABCG1
35 belongs to the ATP binding cassette (ABC) transporter gene family. ABCG1 was
recently published as the human analogue of the drosophila white gene [6-8].

40 25 The gene is strongly upregulated by AcLDL-mediated cholesterol loading, and
almost completely downregulated by HDL₃ mediated-cholesterol deloading, as
45 confirmed by Northern blot (Example 2). Northern blot analysis of mRNA from
human monocyte-derived macrophages obtained from the peripheral blood
30 probands clearly show upregulation of ABCG1 mRNA formation upon AcLDL
incubation. In sharp contrast, ABCG1 mRNA expression was decreased in such
50 macrophages upon incubation with HDL₃ containing medium.

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ABCG1 expression in cholesterol loaded and deloaded cells after four days pre-differentiation

5 For effective cholesterol loading monocytes must be differentiated to phagocytic-macrophage like cells. During this period scavenger receptors are upregulated and promote AcLDL uptake leading to cholestrylo ester accumulation. After four days preincubation period we have incubated the cells for one, two and three days with AcLDL (100 µg/ml) to show cholestrylo ester accumulation. After two days of loading we deloaded the cells with HDL₃ for 12 hours, 24 hours and 48 hours, respectively. ABCG1 is time dependently upregulated during the AcLDL loading period and downregulated by HDL₃ deloading (Examples 2 and 3) In order to confirm time dependent increase of ABCG1 mRNA expression after AcLDL challenge in human monocyte derived macrophages, Nothern blot analyses for ABCG1 mRNA quantification were made, RNA samples from the macrophages were harvested at day zero and day four as controls and mRNA samples were taken one, two, and three days after AcLDL treatment of macrophages, which started at day four. A dramatic increase of ABCG1 mRNA content of the macrophages could be detected from day five through day seven by Nothern blot analyscs.

20

15 This regulation shows the same pattern as changes of cellular cholestrylo ester content (Example3). Cholestrylo ester accumulation starts in monocyte-derived macrophages upon AcLDL stimulation from a base level below 5 nmol/mg cell protein at day four up to 120 nmol/mg cell protein at day seven (i.c. three days after AcLDL application).

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Tissue expression

30 Besides cholesterol loaded macrophages ABCG1 is prominently expressed in brain, spleen, lung, placenta, adrenal gland, thymus and fetal tissues (Table 1).

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Chromosomal location and associated genes and diseases

10 The ABCG1 gene maps to human chromosome 21q 22.3. Also localized in this
region 21q 22.3 are the following genes: integrin β 2 (CD18), brain specific
5 polypeptide 19, down syndrome cell adhesion molecule, dsRNA specific adenosine
deaminase, cystathionine β synthase, collagen VI alpha-2, collagen XVIII alpha-1,
15 autosomal recessive deafness, and amyloid beta precursor.

20 This chromosomal region is in close proximity to other regions involved in Down
10 syndrome, autosomal dominant bipolar affective disorder, and autosomal recessive
non-syndromic deafness.

Extracellular loop of ABCG1 (ABC8) for antibody generation

25 15 The putative structure of the hydrophobic transmembrane region of ABCG1 shows 6
transmembrane spanning domains, and 3 extracellular loops, two of them are 9- and
30 8-amino acids-long, respectively, while the third one is 66-amino acids-long.

35 35 The larger one of the two intracellular loops consists of 30 amino acids. Similarity-
20 survey in protein databases for homologies the 3rd extracellular loop (IIIex) with
other genes resulted in the identification of fibronectin, integrin β 5, RAP, LRP (LDL
receptor related protein) apo-lipoprotein B 100 precursor protein, glutathion S-
40 transferase and glucose transporter.

45 25 A polyclonal antiserum was generated against the 3rd extracellular loop (IIIex) of
ABCG1 in order to perform flow cytometric analysis, energy transfer experiments
and Western-blotting (see Example 3). In the amino acid sequence of ABCG1 the 3rd
extracellular loop (IIIex) comprises 66 amino acids comprises 66 amino acids from
50 30 amino acid 580 through 644. The peptide fragment for antibody generation
comprises the amino acid residues 613 through 628 of ABCG1 polypeptide. ABCG1
obviously interacts with endogenous sequence motifs with other membrane receptors

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involved in transport (e.g. LRP, RAP), signalling and adhesion (e.g. integrins, integrin associated proteins) as a basis of ABCG1-function and regulation. Moreover sequence comparisons of all ABC-transporters listed in Table 3 indicates functional cooperation with other membrane receptors as a general principle of the whole gene family.

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Subfamily-Analysis

Evolutionary relationship studies with the whole ABC transporter family have shown
20 that ABCG1 (ABC8) forms a subfamily together ABCG2 (est157481) and this subfamily is closely related to the full-size transporters ABCA1 (ABC1), ABCA2 (ABC2), ABCA3 (ABC3), ABCA4 (ABCR) and the half-size transporter ABCF1 (TSAP).

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Recent studies by Allikmets et al. have identified 21 new genes as ABC transporters
30 by expressed sequence tags database search [13].

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General description of the ABC transporter family

The ATP-binding cassette (ABC) transporter superfamily contains some of the most
40 functionally diverse proteins known. Most of the members of the ABC family (also called traffic ATP-ases) function as ATP-dependent active transporters (Table 3). The typical functional unit consists of a pair of ATP-binding domains and a set of transmembrane (TM) domains. The TM-domains determine the specificity for the type of molecule transported, and the ATP-binding domains provide the energy to move the molecule through the membrane [14; 15]. The variety of substrates handled by different ABC-transporters is enormous and ranges from ions to peptides. Specific transporters are found for nutrients, endogenous toxins, xenobiotics, peptides, aminoacids, sugars, organic/inorganic ions, vitamins, steroid hormones and drugs [16; 17].

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ABC-transporter associated diseases

10 The search for human disease genes (Table 3) provided a number of previously undiscovered ABC proteins [16]. The best characterized disease caused by a mutation in an ABC transporter is cystic fibrosis (ABCC7 (CFTR)). Inherited disorders of peroxisomal metabolism as Adrenoleukodystrophy and Zellweger's syndrome also show alterations in ABC transporters. They are involved in peroxisomal beta-oxidation, necessary for very long chain fatty acid metabolism [18].

15 20 **Antisense against ABCG1 inhibits cholesterol efflux to HDL₃**

25 30 Since ABCG1 is a cholesterol sensitive gene and other ABC transporters are known to be involved in certain lipid transport processes, the question arises whether ABCG1 plays a role in transport of cholesterol, phospholipids, fatty acids or glycerols. Therefore antisense experiments were performed to test the influence of ABCG1 on lipid loading and deloading. The inhibition of ABCG1 with specific antisense oligonucleotides decreased the efflux of cholesterol and phosphatidyl-choline to HDL₃. (Example 5)

35 40 **Other cholesterol sensitive ABC transporter**

45 Cloning and sequencing of the human ABCA1 (ABC1) provided the information to characterize ABCA1 for cholesterol sensitivity, and tissue distribution (Example 6). Another cholesterol sensitive human ABC transporter (ABCA8) has been cloned and sequenced (Example 8)

50 **Characterization of the ABCG1 promoter region**

55 The ABCG1 promoter has the characteristic binding sites for transcription factors that are involved in the differentiation of monocytes into phagocytic macrophages. The cholesterol sensitivity of the expression of ABCG1 is represented by the transcription factor pattern that is relevant for phagocytic differentiation (Example 7).

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Examples

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Example 15 **Identification of cholesterol loading and deloading candidate genes**

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Monocyte isolation and cell culture

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Monocytes were obtained from peripheral blood of healthy normolipidemic volunteers by leukapheresis and purified by counterflow clutriation. Purity of isolated monocytes was >95% as revealed by FACS analysis. 10x10⁶ monocytes were seeded into 100 mm² diameters cell culture dishes under serum free conditions in macrophage medium for 12 hours in a humidified 37°C incubator maintained with a 5% CO₂, 95% air atmosphere. After 12 hours medium containing unattached cells was replaced by fresh macrophage medium supplemented with 50 ng/ml human recombinant M-CSF (this medium is the standard medium for any further incubations).

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Isolation of lipoproteins and preparation of AcLDL

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Lipoproteins were prepared from human plasma from healthy volunteer donors by standard sequential ultracentrifugation methods in a Beckman L-70 ultracentrifuge equipped with a 70 Ti rotor at 4°C to obtain LDL ($d=1,006$ to $1,063$ g/ml) and HDL₃ ($d=1,125$ to $1,21$ g/ml). All densities were adjusted with solid KBr. Lipoprotein fractions are extensively dialyzed with phosphate-buffered saline (PBS) containing 5 mM EDTA. The final dialysis step was in 0,15 mol/L NaCl in the absence of EDTA. Lipoproteins were made sterile by filtration through a 0.45 µm (pore-size) sterile filter (Sartorius).

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40 LDL was acetylated by repeated addition of acetic anhydride followed by dialysis against PBS [19]. Modified LDL showed enhanced mobility on agarose gel electrophoresis.

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Incubation of monocyte-macrophages with AcLDL and HDL₃

10 After 12 hours of preincubation cells were grown in the presence or absence (control) of 100 µg protein /ml AcLDL for further 3 day in medium. Then, the incubation
5 medium was replaced with fresh medium and incubated with or without the addition of HDL₃ (100 µg/ml) for another 12 hours.

15

Differential display

20 Differential display screening was performed for new candidate genes that are regulated by cholesterol loading/delowering as described [20; 21]. In brief, 0,2 µg of total RNA isolated from monocytes at various incubations was reverse transcribed with specific anchored oligo-dT primers, using a commercially available kit (GeneAmp RNA PCR Core Kit, Perkin Elmer, Germany). The oligo-dT primers used had two additional nucleotides at their 3' end consisting of an invariable A at the
25 second last position (3'-end) and A, C, G or T at the last position to allow a subset of mRNAs to be reverse transcribed. Here, a 13-mer oligo-dT (T101: 5'T11AG-2')
15 was used in a 20-µl reaction at 2,5 µM concentration. One tenth of the cDNA was amplified in a 20-µl PCR reaction using the same oligo-dT and an arbitrary 10-mer upstream primer (D20 5'-GATCAATCGC-3'), 2,5 µM each, using 2,5 units of TAQ
30 DNA Polymerase and 1.25 mM MgCl₂. Amplification was for 40 cycles with denaturation at 94°C for 30 sec, annealing at 41°C for 1 min and elongation at 72°C for 30 sec with a 5 min extension at 72°C following the last cycle. All PCR reactions were carried out in a Perkin Elmer 9600 thermocycler (Perkin Elmer, Germany).
35 PCR-products were separated on ready to use 10% polyacrylamide gels with a 5% stacking gel (CleanGel Large-10/40 ETC, Germany) under non-denaturating conditions using the Multiphor II electrophoresis apparatus (Pharmacia, Germany).
40 The DNA fragments were visualized by silverstaining of the gel as previously described [22].

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Cloning and sequencing of differentially expressed cDNAs

10 cDNA bands of interest were cut out of the gel and DNA was isolated by boiling the
gel slice for 10 min in 20 µl of water. A 4 µl aliquot was used for the following PCR-
reaction in a 20µl volume. The cDNA was reamplified using the same primer set and
15 PCR conditions as above, except, that the final dNTP concentration was 1mM each.
Reamplified cDNAs were cloned in the pUC18-vector using ABCC8 (SUR)eClone-
Kit (Pharmacia), sequenced on an automated fluorescence DNA sequencer using the
AutoRead Sequencing Kit (Pharmacia, Germany) and used as probes for Northern
blot analysis [23].

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Example 2**25 Northern Blot analyses of monocytes and macrophages after 3 days AcLDL
incubation followed by 12 hours HDL₃ incubation**

30 15 Elutriated monocytes were incubated with AcLDL (100 µg/ml medium) for 2.5 days
or differentiated for the same time without the addition of AcLDL as control.
ABCG1 (ABC8) expression is 4 times stronger upregulated with AcLDL incubation
than in differentiated monocytes .After the AcLDL incubation period cells were
washed and incubated with HDL₃ for the next 12 hours or with medium alone as
35 control. ABCG1 expression is almost completely downregulated by HDL₃ incubation
and only moderately decreased in control incubation as confirmed by Northern
blot. For effective cholesterol loading monocytes must be differentiated to macro-
phage like cells. During this period scavenger receptors are upregulated and promote
AcLDL uptake leading to cholestryl ester accumulation. To differentiate the cells
40 prior to AcLDL-dependent cholesterol loading, we cultured the cells for four days in
standard medium. At day four, cells were washed and incubated with AcLDL
(100µg/ml medium) or in the absence of AcLDL as control for further one, two and
45 three days to load the cells with cholesterol. At each timepoint cells were lysed with
0.1 % SDS and lipid was extracted as described in materials and methods and cellular
cholestryl ester was determined by HPTLC-separation. Cells were loaded time
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dependently up to 120 nmol/mg cell protein after 3 days AcLDL loading, whereas in unloaded cells no cholesteryl ester accumulation could be observed.

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To distinguish HDL₃ dependent and independent cholesterol efflux cells were pulsed with AcLDL (100 µg/ml) for three days with the coincubation of ¹⁴C-cholesterol (1,5 µCi/ml medium). Cells were washed and deloaded with HDL₃ (100 µg/ml) for 12 hours, 24 hours and 48 hours, respectively. Cells were incubated without the addition of exogenous lipid-acceptors as a control. After chase period the content of ¹⁴C-cholesterol was determined in the medium and in the cells by liquid scintillation as described in material and methods. The efflux of cholesterol is expressed in percent of cellular DPMs of total DPMs (counts in the cells plus medium) With HDL₃, the efflux is faster and more intense, than the efflux without the addition of HDL₃ as an endogenous lipid acceptor. After 12 hours cellular cholesterol content was reduced to 68 % with HDL₃-dependent deloading, and 86 % in HDL₃-independent deloading. After 48 hours only 35 % of loaded ¹⁴C-cholesterol was observed in the cells treated with HDL₃. In contrast, 70 % of loaded ¹⁴C-cholesterol was found in untreated cells

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25 **Materials:**

Macrophage medium (Macrophage-SFM) was obtained from Gibco Life Technologies, Germany. Human recombinant M-CSF was obtained from Genzyme Diagnostics, Germany, and antisense phosphorothioate oligonucleotides were supplied by Biognostics, Germany. All other chemicals were purchased from Sigma. Nylon membranes and a³²P-dCTP were obtained from Amersham, Germany, ¹⁴C-

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cholesterol and 3H-choline chloride from NEN, Germany, and cell culture dishes are Becton Dickinson, Germany

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Isolation of total RNA and northern blotting

5 Total RNA was isolated at each time-point, before and after AcLDL incubation, and after HDL₃ incubation, respectively. Washed cells were solubilized in guanidine isothiocyanate followed by sedimentation of the extract through cesium chloride [24]. For Northern analysis, 10 µg/lane of total RNA samples were fractionated by electrophoresis in 1,2% agarose agarose gel containing 6% formaldehyde and blotted onto nylon membranes (Schleicher & Schüll, Germany). After crosslinking with UV-irradiation (Stratalinker model 1800, Stratagene, USA), the membranes were hybridized with a cDNA probe for ABCG1 (ABC8). Hybridization and washing conditions were performed as recommended by the manufacturer of the membrane.

20

Example 3

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Westernblot analysis of monocytes and macrophages after cholesterol loading and deloading

35 Protein expression of ABCG1 (ABC8) is upregulated in AcLDL-loaded and down-regulated in HDL₃-deloaded monocyte-derived macrophages. Western blotting with a peptide antibody against ABCG1 as described in materials and methods is performed with 40 µg of total protein for each lane of SDS-PAGE. ABCG1-protein expression is shown in freshly isolated monocytes (day zero) and in differentiated monocytes (day four). From day four to day seven (5d; 6d; 7d) monocyte-derived macrophages were loaded with AcLDL or without AcLDL as control. AcLDL loaded cells from day 6 (6d) were deloaded with HDL₃ for 12, 24, and 48 hours and without exogenous added HDL lipid-acceptor. AcLDL increases the protein-expression, whereas HDL₃ decreases the expression to normal levels again.

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Protein isolation and determination

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At each timepoint cells were lysed with 0.1% SDS and the protein content was determined by the method of Lowry et al. [25].

15

Generation of ABCG1 specific antibodies

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ABCG1 specific peptide antibodies were generated by immunization of chickens and rabbits with a synthetic peptide (Fa. Pineda, Berlin). The peptide sequence was chosen from the extracellular domain exIII amino acid residues 613-628 of ABCG1 comprising the amino acids REDLHCDIDETCHFQ (see sequence listing ID No. 53). After 58 days of immunization western blotting was performed with 1:1000 diluted serum and 1:10000 secondary peroxidase labelled antibody.

25

Electrophoresis and immunoblotting

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SDS-polyacrylamide gelelectrophoresis was performed with 40 μ g total cellular protein per lane. Proteins were transferred to Immobilon as reported. Transfer was confirmed by Coomassie Blue staining of the gel after the electroblot. After blocking for at least 2 hours in 5% nonfat dry milk the blot was washed 3 times for 15 minutes in PBS. Antiserum generated as described was used at 1:1000 dilution in 5% nonfat dry milk in PBS. The blot was incubated for 1 hour. After 4 times washing with PBS at room-temperature a secondary peroxidase-labelled rabbit anti chicken IgG-antibody (1:10000 diluted, Sigma) was incubated in 5% nonfat dry milk in PBS for 1 hour. After 2 times washing with PBS, detection of the immune complexes was carried out with the ECL Western blot detection system (Amersham International PLC, UK).

40

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Fluorescence resonance energy transfer:

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Monocytes were labelled with the specific antibodies for 15 minutes on ice, one antibody is labelled by biotin, the other one is labelled by phycoerythrin. After washing the cells were incubated with a Cy5-conjugated streptavidin for another 15 minutes.

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5 Distances between antibody labelled proteins on the cell surface is measured by
10 energy transfer with a FACScan (Becton Dickinson). Following single laser excitation at 488 nm the Cy5 specific emmission represents an indirect excitation of Cy5
15 dependent on the proximity of the PE-conjugated antibody. The relative transfer efficiency was calculated following standardisation for the intensity of PE and Cy5 labelling and nonspecific overlap of fluorescence based on dual laser excitation and comparison to separately stained control samples.

Example 4

20 **Cholesterol sensitivity of ABCG1 (ABC8) and other members of the ABC-transporter family**
25 The influence of cholesterol loading and deloading on other members of the ABC-family was also investigated to find out the potential second half-size ABC
15 transporter.

30 Further analysis has been performed to examine the expression pattern of all human ABC transporters in monocytes and monocyte derived macrophages as well as in cholesterol loaden and deloaden mononuclear phagocytes.

35 20 The experiments were performed by RT-PCR with cycle-variation to compare the expression in the quantitative part of the distinct PCR. Primer sets were generated from the published sequences of the ABC-transporters. A RT-PCR with GAPDH
40 primers was used as control.

25 45 Several ABC-transporters are also cholesterol sensitive which further supports the function of ABC-transporters in cellular lipid trafficking (Table 2).

Semi-quantitative RT-PCR

30 50 All known ABC-transporters are tested for AcLDL/HDL₃ sensitive regulation of expression using RT-PCR with cycle-variation to compare the expression in the

5 quantitative part of the distinct PCR. 1 µg of total RNA was used in a 40 µl reverse
10 transcription reaction, using the Reverse Transkription System (Promega, Corp. WI,
USA). Aliquots of 5 µl of this RT-reaction was used in 50µl PCR reaction. After
denaturing for 1,5 min at 94°C, 35 or less cycles of PCR were performed with
5 92,3°C for 44s, 60,8°C for 40s (standard annealing temperature differs in certain
15 primer-combinations), 71,5°C for 46s followed by a final 5-min extension at 72°C.
The Primer sets were generated from the published sequences of the ABC-
transporters. A RT-PCR with primers specific for GAPDH was performed as control.

20 The expression pattern of ABC-transporters in monocytes, monocyte derived
macrophages (3 days cultivated monocytes in serum free macrophage-SFM medium
containing 50 ng/ml M-CSF), AcLDL incubated monocytes (3 days with 100 µg/ml)
followed by HDL₃ (100 µg/ml) incubated monocytes is shown in Table 2. Expressed
25 genes are tested for cholesterol sensitivity by semi-quantitative PCR.

15 **Example 5:**

30 **Functional analyses of the cholesterol sensitive ABCG1 (ABC8) transporter
gene by antisense oligonucleotide experiments**

35 Antisense experiments were conducted in order to address the question, that beyond
being regulated by cholesterol loading and deloading ABCG1 is directly involved in
20 lipid loading and deloading processes.

40 In various experiments antisense oligonucleotides decreased the efflux of cholesterol
and phosphatidylcholine to HDL₃. During the loading period with AcLDL the cells
were coincubated with 17 different antisense oligonucleotides. To measure the efflux
of cholesterol and phospholipids the cells were pulsed in the loading period with 1,5
25 µCi/ml ¹⁴C-cholesterol and 3µCi/ml ³H-choline chloride. The medium was changed
and during the chase period cells were incubated with or without HDL₃ for 12 hours.
45 The ¹⁴C-cholesterol and ³H-choline content in the medium and in the cell lysate was
measured and the efflux was determined in percent of total ¹⁴C-cholesterol and ³H-
50 choline loading.

5

10

The most effective antisense oligonucleotide (AS Nr.2) inhibited cholesterol and phospholipids efflux relative to cells that were treated with control antisense (AS control). A dose dependent decrease in cholesterol efflux of 16,79% (5nmol AS) and 32,01% (10 nmol AS) could be shown, respectively.

15

Antisense incubation

20

To inhibit the induction of ABCG1 cells were treated with three different antisense oligonucleotides targeting ABCG1 or one scrambled control-antisense oligonucleotide during the AcLDL-incubation period.

10

Determination of cholesterol and phosphatidylcholine efflux from monocytes in dependency of antisense oligonucleotide treatment

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To measure the efflux of cholesterol and phospholipids the cells were pulsed in addition to AcLDL-incubation with 1,5 µCi/ml ¹⁴C-cholesterol and 3µCi/ml ³H-choline chloride. The medium was changed and in chase period the cells were incubated with or without HDL₃ for 12 hours. Lipid extraction was performed according to the method of Bligh and Dyer [26]. The ¹⁴C-cholesterol and ³H-choline content in the medium and in the cell lysate was measured by liquid scintillation counting and the efflux was determined in percent of total ¹⁴C-cholesterol and ³H-choline loading as described [27]

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Computer analyses

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DNA and protein sequence analyses were conducted using programs provided by HUSAR, Heidelberg, Germany: <http://genius.embnet.dkfz-heidelberg.de:8080>.

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Example 6

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Complete cDNA sequence of the human ATP binding cassette transporter 1 (ABCA1 (ABC1)) and assessing the cholesterol sensitive regulation of ABCA1 mRNA expression.

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cDNA Cloning and Primary Protein Structure

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We have cloned a 6880-bp cDNA containing the complete coding region of the human ABCA1 gene (Figure 8) The open reading frame of 6603 bp encodes a 2201-amino acid protein with a predicted molecular weight of 220 kDa. This protein displays a 94% identity on the amino acid level in an alignment with mouse ABCA1 and can therefore be considered as the human ortholog.

25

Tissue Distribution of ABCA1 mRNA Expression

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In order to examine the tissue-specific expression of ABCA1 a multiple tissue RNA master blot containing poly A⁺ RNA from 50 human tissues was carried out. Northern Blot analysis demonstrates the presence of a ABCA1 specific signal in all tissues. It is mostly prominent in adrenal gland, liver, lung, placenta and all fetal tissues examined so far (Table 1). The weakest signals are found in kidney, pancreas, pituitary gland, mammary gland and bone marrow.

35

Sterol Regulation of ABCA1 mRNA Expression

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In order to determine the regulation of ABCA1 in monocytes/macrophages during cholesterol loading/depletion Northern Blot analysis was performed. The cloned 1000-bp DNA fragment derived from PCR amplification of RNA from five day differentiated monocytes with primers ABCA1 3622f (*CGTCAGCACTCTGATGATGGCCTG-3'*) and ABCA1 4620r (*TCTCTGCTATCTCCAACCTCA-3'*) was hybridized to Northern Blots containing RNA of differentially cultivated monocytes (figure 12) As can be seen in lanes one to five, the ABCA1 mRNA is increased during in vitro differentiation of freshly isolated monocytes until day five. Longer cultivation results in a total loss of

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5 expression. When the cells were incubated in the presence of AcLDL to induce sterol
10 loading (lanes 6-8) beginning at day four, a much stronger accumulation of mRNA
can be detected in comparison to control cells (lanes 2-5). When these cells were
cultured with HDL₃ as cholesterol acceptor for 12h, 24h and 48h (lanes 9-11) the
15 ABCA1 signal significantly decreases with respect to control cells incubated in the
absence of HDL₃ (lanes 12-14). Taken together, these results indicate that ABCA1 is
a sterol-sensitive gene which is induced by cholesterol loading and downregulated by
cholesterol depletion.

20 Cell culture.

10 Peripheral blood monocytes were isolated by leukaphresis and counterflow
elutriation (19JBC). To obtain fractions containing >90% CD 14 positive
25 mononuclear phagocytes, cells were pooled and cultured on plastic Petri dishes in
macrophage SFM medium (Gibco BRL) containing 25 U/ml recombinant human M-
CSF (Genzyme) for various times in 5% CO₂ in air at 37°C. The cells were incubated
15 in the absence (differentiation control) or presence of AcLDL (100 µg/ml) to induce
sterol loading. Following this incubation the cells were cultured in fresh medium
supplemented with or without HDL₃ (100 µg/ml) for additional times in order to
achieve cholesterol efflux from the cells to its acceptor HDL₃.

35 Preparation of RNA and Northern blot analysis.

20 Total cellular RNA was isolated from the cells by guanidium isothiocyanate lysis and
40 CsCl centrifugation (Chirgwin). The RNA isolated was quantitated
spectrophotometrically and 15 µg samples were separated on a 1.2% agarose-
formaldehyde gel and transferred to a nylon membrane (Schleicher & Schüll). After
45 crosslinking with UV-irradiation (Stratalinker model 1800, Stratagene), the
membranes were hybridized with a 1000 bp DNA fragment derived from PCR
25 amplification with primers ABCA1 3622f and ABCA1 4620r, stripped and
subsequently hybridized with a human β-actin probe. In order to determine the
tissue-specific expression of ABCA1 a multiple tissue RNA master blot containing

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poly A⁺ RNA from 50 human tissues was purchased from Clontech. The probes were radiolabeled with [γ -³²P]dCTP (Amersham) using the Oligolabeling kit from Pharmacia. Hybridization and washing conditions were performed following the method described previously (Virca).

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5 cDNA cloning of human ABCA1

Based on sequence information of mouse ABCA1 cDNA we designed primers for RT-PCR analysis in order to amplify the human ABCA1 (ABCI) cDNA. Approximately 1 μ g of RNA from five day differentiated mononuclear phagocytes was reverse transcribed in a 20 μ l reaction using the RNA PCR Core Kit from Perkin Elmer. An aliquot of the cDNA was used in a 100 μ l PCR reaction performed with AmpliTaq Gold (Perkin Elmer) and the following primer combinations: (primer names indicate the position in the corresponding mouse cDNA sequence):

30 15 *mABC1-144f* (5'-CAAACATGTCAGCTGTTACTGGA-3') and

mABC1-643r (5'-TAGCCTTGCAAA-AATACCTTCTG-3'),

35 15 *mABC1-1221f* (5'-GTTGGAAAGATTCTCTATAACACCTG-3') and

mABC1-1910r (5'-CGTCAGCACTCTGATGATGGCCTG-3'),

40 20 *mABC1-3622f* (5'-TCTCTGCTATCTCCAACCTCA-3') and

mABC1-4620r (5'-ACGTCTTCACCAGGTAATCTGAA-3'),

45 25 *mABC1-5056f* (5'-CTATCTGTGTCATCTTGCGATG-3') and

mABC1-5857r (5'-CGCTTCCTCCTATAGATCTTGGT-3'),

50 30 *mABC1-6093f* (5'-AAGAGAGCATGTGGA-GTTCTTG-3') and

mABC1-7051r (5'-CCCTGTAATGGAATTGTGTTCTC-3'),

35 35 *hABC1-540f* (5'-AACCTTCTCTGGGTTCCTGTATC-3') and

hABC1-1300r (5'-AGTTCTGGAA-GGTCTTGTTCAC-3'),

40 40 *hABC1-1831f* (5'-GCTGACCCCTTGAGGACATGCG-3') and

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5

- hABC1-3701r* (5'-ATAGGTCAGCTCATGCCCTATGT-3'),
10 *hABC1-4532f* (5'-GCTGCC-TCCTCCACAAAGAAAAC-3') and
hABC1-5134r (5'-GCTTGCTGACCCGCTCC-TGGATC-3').
15 *hABC1-5800f* (5'-GAGGCCAGAATGACATCTTAGAA-3') and
hABC1-6259r (5'-CTTGACAACACTTAGGGCACAAAT-3').

15

20 All PCR products were cloned into the pUC18 plasmid vector and the nucleotide sequences were determined on a Pharmacia ALFexpress sequencer using the dideoxy chain-termination method and fluorescent dye-labeled primers.

25

Example 7

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Identification of the 5'end of ABCG1

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15 We could partially prove the 5'-end of ABCG1 published by Chen [7] that differs from the 5'-end published by Croop [6] obtained from the mRNA of human monocytes/macrophages using a 5' RACE approach. In detail the sequence according to Chen et al. downstream of position 25 was in agreement with our own data. In contrast, our identified sequence differs from the one reported by Chen [7] and Croop [6] at a site upstream of position 25 (Chen [7]). The sequence SEQ ID NO: 32 shows the newly identified 5'-end followed by the sequence published by Chen [7] from position 25.

40

45

Molecular cloning and characterisation of the ABCG1 5'UTR

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25 We identified several fragments by screening of a λ phage library which contained a total of app. 3 kb of the 5' UTR upstream sequence of the human ABCG1 gene. The

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sequence that comprises the 5'UTR and part of exon 1 (described above) are given in SEQ ID NO: 54.

10

The promoter activity of this sequence was proven by luciferase reporter gene assays in transiently transfected CHO cells.

15

Putative transcription factor binding sites within the promoter region with the highest likelihood ratio for the matched sequence as deduced from the TransFac database, GFB, Braunschweig, Germany. Multiple binding sites for SP-1, AP-1, AP-2 and CCAAT-binding factor (C/EBP family) are present within the first 1 kb of the putative promoter region.

20

10 Additionally, a transcription factor binding site involved in the regulation of apolipoprotein B was identified.

25

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Example 8

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Characterization of the human ABCA8 full length cDNA

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The putative ABCA8 coding sequence is app. 6.5 kb in size. We successfully cloned and sequenced a 1kb segment of the human ABCA8 cDNA that encodes the putative second nucleotide binding site of the mature polypeptide (the sequence is shown in the sequence listing). The nucleotide sequence exhibits a 73% homology with the known human ABCA1 (ABC1) cDNA sequence.

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We identified an alternative transcript in the cloned 1 kb coding region which consists of a 72 bp segment (see sequence listing). Genomic analysis of this region revealed that the alternative sequence is identical with a complete intron suggesting that the alternative mRNA is generated by intron retention. The retained intron introduces a preterminal stop codon and thus may code for a truncated ABCA8 variant.

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ABCA8 also shows a cholesterol sensitive regulation of the mRNA expression
10 (Table 2).

5 Tissue expression of ABCA8 is shown in table 1.

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Example 9

Characterisation of the regulation of ABC transporter during differentiation of 20 keratinocytic cells (HaCaT)

Differentiation of epidermal keratinocytes is accompanied by the synthesis of
25 specific lipids composed mainly of sphingolipids (SL), free fatty acids (FFA),
cholesterol (CH), and cholesterol sulfate, all involved in the establishment of the
epidermal permeability barrier. The skin and, in particular, the proliferating layer of
15 the epidermis is one of the most active sites of lipid synthesis in the entire organism.
Cholesterol synthesis in normal human epidermis is LDL-independent, and circu-
30 lating cholesterol levels do not affect the cutaneous de novo cholesterol synthesis.
Fully differentiated normal human keratinocytes lack LDL receptors or its expression
is very low, whereas in the normal human epidermis only basal cells express LDL
20 receptors.

35

During keratinocyte differentiation a shift from polar glycerophospholipids to neutral
40 lipids (FFA, TG) and also a replacement of short chain FFA by long chain highly
saturated FFA is observed. The most important lipids for the barrier function of the
25 skin are sphingolipids that account for one third of the lipids in the cornified layer,
and consist of a large ceramide fraction as a result of glucosylceramide degradation
45 by intercellular glycosidases and de novo synthesis of ceramide.

Glucosylceramide is synthesized intracellularly and stored in lamellar bodies and
50 glucosylceramide synthase expression was found up-regulated during the differen-
tiation of human keratinocytes.

55

5

10 Cholesterol sulfate is formed by the action of cholesterol sulfotransferase during keratinocyte differentiation . Cholesterol sulfate and the degrading enzyme steroid sulfatase are present in all viable epidermal layers, with the highest levels in the
5 stratum granulosum. The gradient of cholesterol sulfate content across the stratum corneum (from inner to outer layers), and progressive desulfation of cholesterol sulfate regulate cell cohesiveness and normal stratum corneum keratinization and desquamation, respectively. Cholesterol sulfate induces transglutaminase 1 and the coordinate regulation of both factors is essential for normal keratinization .

20

10 The final step in lipid barrier formation involves lamellar body secretion and the subsequent post-secretory processing of polar lipids into their nonpolar lipid products through the action of hydrolytic enzymes that are simultaneously released (β -glucocerebrosidase, phospholipases, steroid sulfatase, acid sphingomyelinase).
25 15 Disruption of the permeability barrier results in an increased cholesterol, fatty acid, and ceramide synthesis in the underlying epidermis. It has been shown that mRNA levels for the key enzymes required for cholesterol, fatty acid, and ceramide synthesis increased rapidly after artificial barrier disruption .

30

35 20 Currently the lipid transport systems in keratinocytes are poorly characterized. Several fatty acid transport related proteins have been identified in keratinocytes: plasma membrane fatty acid transport proteins (FATPs) and intracellular fatty acid binding proteins (FABPs), most of them exhibiting high affinity for essential fatty acids. The expression of epidermal FABPs is up-regulated in hyperproliferative and
40 25 inflammatory skin diseases, during keratinocyte differentiation and barrier disruption

45

30 Based on our data on macrophages, we propose several ABC transporters as putative candidates for cellular lipid export in keratinocytes. We have examined the expression of all known ABC transporters during HaCaT cells differentiation. The human
50 55 HaCaT cell line has a full epidermal differentiation capacity. Keratinocytes grown in

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vitro as a monolayer at low calcium concentration (< 0.1 mM) can be differentiated by increasing calcium concentration in the culture medium (1-2 mM). We cultured HaCaT cells as a monolayer in calcium-free RMPI (Gibco) medium mixed with standard Ham's F12 medium at a ratio 3:1 supplemented with 10% chelex-treated FCS, Penicillin and Streptomycin. The final concentration of calcium in above medium was 0.06 mM. When the cells reached confluence (usually on 5th day of the culture), calcium concentration was enhanced up to the level of 1.2 mM. The cells were seeded at a density of $2 \times 10^5 / \text{cm}^2$ in 60 mm culture dishes. The culture medium was replaced every two day and the cells were harvested after 24 h, 48h h, 4 d, 6 da, 8 d and 10 d in culture, respectively. Total RNA from HaCaT cells was isolated using the isothiocyanate/cesium chloride-ultracentrifugation method.

The expression of all known human ABC transporters was examined during HaCaT cell differentiation (24 h, 48 h, 4 d, 6 d, 8 d, 10d, respectively) using a semi-quantitative RT-PCR approach (Table 6). The primer sets were generated from the published sequences of the ABC-transporters. Primers specific for GAPDH were used as a control. As a marker of keratinocyte differentiation CGT (ceramide glucosyl transferase) gene expression was assessed. Three of the transporters examined, ABCB1 (MDR1), ABCB4 (MDR3), ABCD3 (PMP70), were not expressed. ABCC6 (MRP6), ABCA1 (ABC1),ABCD2 (ALDR and ABCB9 (est122234) were expressed at low levels (Table 6)

Most of the other transporters exhibited a biphasic expression pattern or were downregulated during keratinocyte differentiation. There was, however, a high expression of ABCG1 (ABC8), ABCA8 (new) and ABCC3 (MRP3) indicative for their involvement in terminal keratinocyte lipid secretion for cholesterol, FFAs and ceramide-backbone lipids.. The two peroxisomal ABC transporters, ABCD2 (ALDR) and ABCD1 (ALDP) that mediate the transport of very long chain fatty acids into peroxisomes were initially expressed at relatively low levels and subsequently downregulated during differentiation. This is in agreement with the replacement of

5

short chain fatty acids by very long chain fatty acids during keratinocyte differentiation.

10

Example 10:

15 5 Sequencing of ABCA1 cDNA and genomic structure in five families of patients with Tangier disease revealed different mutations in the ABCA1 gene locus. These patients have different mutations at different positions in the ABCA1 gene, that result in changes in the protein structure of ABCA1. Family members that are heterozygous for these mutations show lowered levels of serum HDL, whereas the
20 10 homoczygote patients have extremely reduced HDL serum levels.

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Claims

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Claims:

- 10 1. A polynucleotide comprising a member selected from the group consisting of:
 - 5 (a) a polynucleotide encoding the polypeptide as set forth in SEQ ID NO:2;
 - (b) a polynucleotide capable of hybridizing to and which is at least 70% identical to the polynucleotide of (a); and
 - (c) a polynucleotide fragment of the polynucleotide of (a) or (b).
- 15 2. The polynucleotide of claim 1 wherein the polynucleotide is DNA.
- 20 3. A vector containing one or more of the polynucleotides of claim 1 and 2.
- 25 4. A host cell containing the vector of claim 3.
- 30 5. A process for producing a polypeptide comprising: expressing from the host cell of claim 4 the polypeptide encoded by said DNA.
- 35 6. A polypeptide selected from the group consisting of
 - 40 (a) a polypeptide having the deduced amino acid sequence of SEQ ID NO:2 and fragments, analogs and derivatives thereof, and
 - (b) a polypeptide comprising amino acid 1 to amino acid 2201 of SEQ ID NO:2.
- 45 7. An antibody capable to bind to the polypeptide of claim 6.
- 50 8. A diagnostic kit for the detection of the polypeptide of claim 6.

5

9. Use of a polypeptides encoded by a polynucleotide comprising a member selected from the group consisting of:

10

- (a) a polynucleotide as set forth in SEQ ID NO:1, 3, 4 and 6 to 31;
- (b) a polynucleotide capable of hybridizing to and which is at least 70% identical to the polynucleotide of (a); and
- (c) a polynucleotide fragment of the polynucleotide of (a) or (b)

15

in an assay for detecting modulators of said polypeptides.

20

10. Modulator of a polypeptides encoded by a polynucleotide comprising a member selected from the group consisting of:

25

- (a) a polynucleotide as set forth in SEQ ID NO:1, 3, 4 and 6 to 31;
- (b) a polynucleotide capable of hybridizing to and which is at least 70% identical to the polynucleotide of (a); and
- (d) a polynucleotide fragment of the polynucleotide of (a) or (b)

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11. A pharmaceutical comprising the modulator of claim 10

35

20. 12. An assay for detecting polypeptides encoded by a polynucleotide comprising a member selected from the group consisting of:

40

- (a) a polynucleotide as set forth in SEQ ID NO:1, 3, 4 and 6 to 32 and 54;
- (b) a polynucleotide capable of hybridizing to and which is at least 70% identical to the polynucleotide of (a); and
- (c) a polynucleotide fragment of the polynucleotide of (a) or (b)

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Figure 1

2588 GA TCAATCGCAT TCATTTAAG AAATTATACC TTTTAGTAC TTGCTGAAGA
 2641 ATGATTCAAG GTAAATACA TACTTGTT AGAGAGGCAGA GGGGTTAAC CCGAGTCACC
 2701 CAGCTGGTCT CATACTAGA CAGCACTGT GAAGGATTGA ATGCAGGTTC CAGGTGGAGG
 2761 GAAGACGTGG ACACCATCTC CACTGAGCCA TGCGAGACATT TTTAAAAGCT ATACACAAAAA
 2821 TTGTGAGAAG ACATTGCCA ACTCTTCMA AGTCTTCTT TTTCCACGTG CTTCTTATTT
 2881 TAAGCGAAAT ATATTGTTG TTTCTCCTA AAAAAAAA 2890

Figure 2

1 CAAACATGTCAGCTGTTACTGGAAGTGGCCTGGCCTCTATTTATCTTCCTGATCCTGATC 60
 61 TCTGTTGGCTGAGCTACCCACCCTATGAACAACTGAATGCCATTTCCTTCAAATAAGCC 120
 121 ATGCCCTCTGCAGGAACACTTCCTGGGTTCAAGGGATTATCTGTAATGCCAACCCCC 180
 1 M P S A G T L P W V Q G I I C N A N N P 20
 181 TGTTTCCGTTACCCGACTCCTGGGAGGCTCCGGAGTTGGAAACTTAAACAAATCC 240
 21 C F R Y P T P G E A P G V V G N F N K S 40
 241 ATTGTGGCTGCCCTGTTCTCAGATGCTGGAGGCTCTTATACAGCCAGAAAGACACC 300
 41 I V A R L F S D A R R L L L Y S Q K D T 60
 301 AGCATGAAGGACATGCCAAAGTCTGAGAACATTACAGCAGATCAAGAAATCCAGCTCA 360
 61 S M K D M R K V L R T L Q Q I K K S S S 80
 361 AACTTGAAGCTTCAAGATTCTGGGACAATGAAACCTCTCTGGGTTCTGTATCAC 420
 81 N L K L Q D F L V D N E T F S G F L Y H 100
 421 AACCTCTCTCTCCCAAAGTCTACTGTGGACAAGATGCTGGGCTGATGTCAATTCTCCAC 480
 101 N L S L P K S T V D K M L R A D V I L H 120
 481 AAGGTATTTGCAAGGCTACCACTACATTGACAAGTCTGTGCAATGGATCAAAATCA 540
 121 K V F L Q G Y Q L H L T S L C N G S K S 140
 541 GAAGAGATGATTCAACTTGGTGACCAAGAAGTTCTGAGCTTGTGGCTACCAAGGGAG 600
 141 E E M I Q L G D Q E V S E L C G L P R E 160
 601 AAACTGGCTGCAGCAGAGCAGTACTCGTCCAAACATGGACATCCTGAAGCCAATCTG 660
 161 K L A A A E R V L R S N M D I L K P I L 180
 661 AGAACACTAAACTCTACATCTCCCTCCCGAGCAAGGAGCTGGCCGAAGCCACAAAAACA 720
 181 R T L N S T S P F P S K E L A E A T K T 200
 721 TTGCTGCATAGTCTTGGACTCTGGCCAGGAGCTGTTCAACATGAGAGCTGGAGTGAC 780
 201 L L H S L G T L A Q E L F S M R S W S D 220
 781 ATGGCACAGGAGGTGATTTCTGACCAATGTGAACAGCTCCAGCTCCACCCAAATC 840
 221 M R Q E V M F L T N V N S S S S S T Q I 240
 841 TACCAAGGCTGTGTCTCGTATTGTCTGGGGCATCCCGAGGGAGGGGGGGCTGAAGATCAAG 900
 241 Y Q A V S R I V C G H P E G G G L K I K 260
 901 TCTCTCAACTGGTATGAGGACAACAACACAAAGCCCTTTGGAGGCAATGGCACTGAG 960
 261 S L N W Y E D N N Y K A L F G G N G T E 280

961 GAAGATGCTGAAACCTTCTATGACAACACTACAACACTCCTTACTGCAATGATTTGATGAAG 1020
281 E D A E T F Y D N S T T P Y C N D L M K 300
1021 AATTTGGAGTCTAGTCCCTTTCCCGCATTATCTGGAAAGCTCTGAAGCCGCTGCTCGTT 1080
301 N L E S S P L S R I I W K A L K P L L V 320
1081 GGGAACATCCTGTATACACCTGACACTCCAGCCACAAGGCAGGTATGGCTGAGGTGAAC 1140
321 G K I L Y T P D T P A T R Q V M A E V N 340
1141 AAGACCTTCAGGAACACTGGCTGTGTTCCATGATCTGGAAAGGCATGTGGGAGGAACCTCAGC 1200
341 K T F Q E L A V F H D L E G M W E E L S 360
1201 CCCAACATCTGGACCTTCATGGAGAACAGCCAAGAAATGGACCTTGTCCGGATGCTGTTG 1260
361 P K I W T F M E N S Q E M D L V R M L L 380
1261 GACAGCAGGGACAATGACCACTTTGGAACAGCAGTTGGATGGCTTAGATTGGACAGCC 1320
381 D S R D N D H F W E Q Q L D G L D W T A 400
1321 CAAGACATCGTGGCGTTTTGGCCAAGCACCCAGAGGATGTCCAGTCCAGTAATGGTTCT 1380
401 Q D I V A F L A K H P E D V Q S S N G S 420
1381 GTGTACACCTGGAGAGAACAGCTTCAACCGAGACTAACCGAGCAATCCGACCATACTCGC 1440
421 V Y T W R E A F N E T N Q A I R T I S R 440
1441 TTCACTGGAGTGTGTCAACCTGAACAGCTAGAACCCATAGCAACAGAAGTCTGGCTCATC 1500
441 F M E C V N L N K L E P I A T E V W L I 460
1501 AACAAAGTCCATGGAGCTGCTGGATGAGAGGAACCTCTGGCTGGTATTGTGTTCACTGGA 1560
461 N K S M E L L D E R K F W A G I V F T G 480
1561 ATTACTCCAGGCAGCATTGAGCTGCCCATCATGTCAGTACAAGATCCGAATGGACATT 1620
481 I T P G S I E L P H H V K Y K I R M D I 500
1621 GACAATGTGGAGAGGACAAATAAAATCAAGGATGGGTACTGGGACCCCTGGCTCTCGAGCT 1680
501 D N V E R T N K I K D G Y W D P G P R A 520
1681 GACCCCTTGAGGACATGCCGTACGCTCTGGGGGGCTTCGCCTACTTGCAGGATGTGGTG 1740
521 D P F E D M R Y V W G G F A Y L Q D V V 540
1741 GAGCACCCAAATCATCAGGGTGCTGACGGGACCCAGAGAAAGAAACTGGTGTCTATATGCAA 1800
541 E Q A I I R V L T G T E K K T G V Y M Q 560
1801 CAGATGCCCTATCCCTGTTACGTTGATGACATCTTCTGGGGGTGATGAGCCGGTCAATG 1860
561 Q M P Y P C Y V D D I F L R V M S R S M 580
1861 CCCCTCTTCATGACGCTGGCTGGATTTACTCAGTGGCTGTGATCATCAAGGGCATCGTG 1920
581 P L F M T L A W I Y S V A V I I K G I V 600
1921 TATGAGAACGGAGGCACGGCTGAAAGAGACCATGCCGATCATGGGCTGGACAACAGCATH 1980
601 Y E K E A R L K E T M R I M G L D N S I 620
1981 CTCTGGTTAGCTGGTTCAATTAGTAGCCTCATTCCCTTCTGTGAGCGCTGGCTGCTA 2040
621 L W F S W F I S S L I P L L V S A G L L 640
2041 GTGGTCATCCTGAAGTTAGGAAACCTGCTGCCCTACAGTGTACCTCCAGCGTGGTGTGTC 2100
641 V V I L K L G N L L P Y S D P S V V F V 660

2101 TTCCCTGTCCGTGTTGCTGTGGTGACAATCCTGCAGTGCTTCTGATTAGCACACTCTTC 2160

661 F L S V F A V V T I L Q C F L I S T L F 680
 2161 TCCAGAGCCAACCTGGCAGCAGGCTGTGGGGCATCATCTACTTCACGCCGTACCTGCC 2220
 681 S R A N L A A A C G G I I Y F T L Y L P 700
 2221 TACGTCCCTGTGTGGCATGGCAGGACTACGTGGCTTCACACTCAAGATCTCGCTAGC 2280
 701 Y V L C V A W Q D Y V G F T L K I F A S 720
 2281 CTGCTGTCCTGTGGCTTTGGCTGTGAGTACTTGCCTTTTGAGGAGCAG 2340
 721 L L S P V A F G F G C E Y F A L F E E Q 740
 2341 GGCATTGGAGTGCAGTGGACAACTGTTGAGAGTCCTGTGGAGGAAGATGGCTCAAT 2400
 741 G I G V Q W D N L F E S P V E E D G F N 760
 2401 CTCACCACTCGGTCTCCATGATGCTGTTGACACCTCCCTATGGGTGATGACCTGG 2460
 761 L T T S V S M M L F D T F L Y G V M T W 780
 2461 TACATTGAGGCTGTCTTCCAGGCCAGTACGGAATTCCAGGCCCTGGTATTTCCCTGG 2520
 781 Y I E A V F P G Q Y G I P R P W Y F P C 800
 2521 ACCAAGTCCACTGTTGGCGAGGAAAGTGAAGTGAAGAGGCCACCCCTGGTCCAACCAG 2580
 801 T K S Y W F G E E S D E K S H P G S N Q 820
 2581 AAGAGAATATCAGAAATCTGCATGGAGGAGGAACCCACCCACTTGAAGCTGGCGTGTCC 2640
 821 K R I S E I C M E E E P T H L K L G V S 840
 2641 ATTCAAGAACCTGGTAAAGTCTACCGAGATGGGATGAAGTGGCTGTCGATGGCTGGCA 2700
 841 I Q N L V K V Y R D G M K V A V D G L A 860
 2701 CTGAATTTTATGAGGCCAGATCACCTCCCTGGCCACATGGAGCGGGGAAGACG 2760
 861 L N F Y E G Q I T S F L G H N G A G K T 880
 2761 ACCACCATGTCAATCTGACCGGGTTGTTCCCCCGACCTCGGGCACCGCTACATCCCTG 2820
 881 T T M S I L T G L F P P T S G T A Y I L 900
 2821 GGAAAAGACATCGCTGAGATGAGCACCATCCGGCAGAACCTGGGGCTGTCCCCAG 2880
 901 G K D I R S E M S T I R Q N L G V C P Q 920
 2881 CATAACGTGCTGTTGACATGCTGACTGTCGAAGAACACATCTGGTTCTATGCCGCTTG 2940
 921 H N V L F D M L T V E E H I W F Y A R L 940
 2941 AAAGGGCTCTCTGAGAACGTGAAGGCGGAGATGGAGCAGATGGCCCTGGATGTTGGT 3000
 941 K G L S E K H V K A E M E Q M A L D V G 960
 3001 TTGCCATCAAGCAAGCTGAAAGCAAACAAGCCAGCTGTCAGGTGGAATGCAGAGAAAG 3060
 961 L P S S K L K S K T S Q L S G G M Q R K 980
 3061 CTATCTGCCCCCTGGCTTGTGGGGATCTAAGGTTGTCATTCTGGATGAACCCACA 3120
 981 L S V A L A F V G G S K V V I L D E P T 1000
 3121 GCTGGTGGACCCCTACTCCCGCAGGGGAATATGGGAGCTGCTGCTGAAATACCGACAA 3180
 1001 A G V D P Y S R R G I W E L L L K Y R Q 1020
 3181 GGCCGCACCAATTATTCTCTACACACCACATGGATGAAGCGGACGTCCCTGGGGACAGG 3240
 1021 G R T I I L S T H H M D E A D V L G D R 1040
 3241 ATTGCCATCATCTCCATGGGAAGCTGTGCTGTGGCTCCCTGGTAAAGAAC 3300
 1041 I A I I S H G K L C C V G S S L F L K N 1060
 3301 CAGCTGGGAACAGGCTACTACCTGACCTGGTCAAGAAAGATGTGGAATCCCTCCAGT 3360

1061 Q L G T G Y Y L T L V K K D V E S S L S 1080
 3361 TCCTGCAGAACAGTAGTAGCACTGTGTCATACTGAAAAAGGAGGACAGTGTTCAG 3420
 1081 S C R N S S S T V S Y L K K E D S V S Q 1100
 3421 AGCAGTTCTGATGCTGCCCTGGCGAGCGACCATGAGAGTGACACGCTGACCATCGATGTC 3480
 1101 S S S D A G L G S D H E S D T L T I D V 1120
 3481 TCTGCTATCTCCAACCTCATCAGGAAGCATGTGTCAGAAGCCCCGCTGGTGGAAAGACATA 3540
 1121 S A I S N L I R K H V S E A R L V E D I 1140
 3541 GGGCATGAGCTGACCTATGTGCTGCCATATGAAGCTGCTAAGGAGGGAGCCTTGTGGAA 3600
 1141 G H E L T Y V L P Y E A A K E G A F V E 1160
 3601 CTCTTTCATGAGATTGATGACCGGCCTCAGACCTGGCATTCTAGTTATGGCATCTCA 3660
 1161 L F H E I D D R L S D L G I S S Y G I S 1180
 3661 GAGACGACCCCTGGAAGAAATATTCTCAAGGTGCCGAAGAGAGTGGGGTGGATGCTGAG 3720
 1181 E T T L E E I F L K V A E E S G V D A E 1200
 3721 ACCTCAGATGGTACCTTGCAGCAAGACGAAACAGGGCCCTTCGGGGACAAGCAGAGC 3780
 1201 T S D G T L P A R R N R R A F G D K Q S 1220
 3781 TGTCTCGCCCGTTCACTGAAGATGATGCTGCTGATCAAATGATTCTGACATAGACCCA 3840
 1221 C L R P F T E D D A A D P N D S D I D P 1240
 3841 GAATCCAGAGAGACAGACTTGCTCAGTGGATGGCAAGGGTCTACCAGGTGAAA 3900
 1241 E S R E T D L L S G M D G K G S Y Q V K 1260
 3901 GGCTGAAACTTACACAGCAACAGTTGTGGCCCTTTGTGAAAGAGACTGCTAATTGCC 3960
 1261 G W K L T Q Q Q F V A L L W K R L L I A 1280
 3961 AGACGGAGTCGGAAAGGATTTTGCTCAGATTGCTTGCAGCTGTGTTGTGCATT 4020
 1281 R R S R K G F F A Q I V L P A V F V C I 1300
 4021 CCCCTTGTGTTCACTGCTGATCGTGCACCCCTTGGCAAGTACCCCAGCCTGGAAACTTCAG 4080
 1301 A L V F S L I V P P F G K Y P S L E L Q 1320
 4081 CCCTGGATGTACAACGAACAGTACACATTGTCAGCAATGATGCTCCTGAGGACACGGGA 4140
 1321 P W M Y N E Q Y T F V S N D A P E D T G 1340
 4141 ACCCTGAACTCTTAAACGCCCTCACCAAGACCCCTGGCTTGGGACCCGCTGTATGGAA 4200
 1341 T L E L L N A L T K D P G F G T R C M E 1360
 4201 GGAAACCCAATCCCAGACACGCCCTGCCAGGCAGGGAGGAAGAGTGGACCACTGCCCA 4260
 1361 G N P I P D T P C Q A G E E E W T T A P 1380
 4261 GTTCCCCAGACCATCATGGACCTCTCCAGAATGGAACTGGACAATGCCAGAACCTTCA 4320
 1381 V P Q T I M D L F Q N G N W T M Q N P S 1400
 4321 CCTGCATGCCACTGTAGCAGCGACAAAATCAAGAAGATGCTGCCGTGTGCCCCCAGGG 4380
 1401 P A C Q C S S D K I K K M L P V C P P G 1420
 4381 GCAGGGGGCTGCCCTCCACAAAAGAAAACAAACACTGCAGATATCCTCAGGACCTG 4440
 1421 A G G L P P P Q R K Q N T A D I L Q D L 1440
 4441 ACAGGAAGAACATTGGATTATCTGGTAACAGTATGTGAGATCATAGCCAAAGC 4500
 1441 T G R N I S D Y L V K T Y V Q I I A K S 1460
 4501 TTAAAGAACAAAGATCTGGGTGAATGAGTTAGGTATGGCGGCTTTCCCTGGGTGTCAGT 4560

1461 L K N K I W V N E F R Y G G F S L G V S 1480
4561 AATACTCAAGCACTTCTCCGAGTCAGAAGTTAATGATGCCACAAACAAATGAAGAAA 4620
1481 N T Q A L P P S Q E V N D A T K Q M K K 1500
4621 CACCTAAAGCTGGCCAAGGACAGTCTGCAGATCGATTCTCAACAGCTGGGAAGATT 4680
1501 H L K L A K D S S A D R F L N S L G R F 1520
4681 ATGACAGGACTGGACACCAGAAAATGTCAAGGTGTGGTTCAATAACAAGGGCTGGCAT 4770
1521 M T G L D T R N N V K V W F N N K G W H 1540
4741 GCAATCAGCTCTTCTGAATGTCAACAATGCCATTCTCCGGGCAACCTGCCAAAAG 4800
1541 A I S S F L N V I N N A I L R A N L Q K 1560
4801 GGAGAGAACCCCTAGCCATTATGAAATTACTGCTTCATCACCCCTGAATCTCACCAAG 4860
1561 G E N P S H Y G I T A F N H P L N L T K 1580
4861 CAGCAGCTCTCAGAGGTGGCTCCGATGACCACATCAGTGGATGTCCTTGTGTCCATCTGT 4920
1581 Q Q L S E V A P M T T S V D V L V S I C 1600
4921 GTCATCTTGCAATGTCCTTCGTCCAGCCAGCTTGTCTGATTCCGTATCCGTGATCCAGGAGCGG 4980
1601 V I F A M S F V P A S F V V F L I Q E R 1620
4981 GTCAGCAAAGCAAAACACCTGCAGTCATCAGTGGAGTGAACCTGTCTACTGGCTC 5040
1621 V S K A K H L Q F I S G V K P V I Y W L 1640
5041 TCTAATTTGCTGGGATATGTGCAATTACGTTGTCCTGCCACACTGGTCATTATCATC 5100
1641 S N F V W D M C N Y V V P A T L V I I I 1660
5101 TTCACTGCTTCCAGCAGAAGTCCTATGTGTCCTCCACCAATGTCCTGTGCTAGCCCTT 5160
1661 F I C F Q Q K S Y V S S T N L P V L A L 1680
5161 CTACTTTGCTGTATGGTGGTCAATCACACCTCTCATGTACCCAGCCTCCCTTGTGTT 5220
1681 L L L Y G W S I T P L M Y P A S F V F 1700
5221 AAGATCCCCAGCACGCTATGTTGCTCACAGCGTGAACCTCTTCATTGGCATTAAAT 5280
1701 K I P S T A Y V V L T S V N L F I G I N 1720
5281 GGCACCGTGGCCACCTTGTGCTGGAGCTGTCACCGACAATAAGCTGAATAATATCAAT 5340
1721 G S V A T F V L E L F T D N K L N N I N 1740
5341 GATATCCTGAAGTCCGTGTTCTGATCTCCCACATTTGCTGGACGAGGGCTCATC 5400
1741 D I L K S V F L I F P H F C L G R G L I 1760
5401 GACATGGTAAAAACCAAGCAATGGCTGATGCCCTGGAAAGCTTGGGGAGAATCGCTT 5460
1761 D M V K N Q A M A D A L E R F G E N R F 1780
5461 GTGTCACCATTATCTGGGACTTGGTGGACGAAACCTCTGCCATGGCGTGGAGGG 5520
1781 V S P L S W D L V G R N L F A M A V E G 1800
5521 GTGGTGTCTCTCATTACTGTTCTGATCCAGTACAGATTCTCATCAGGCCAGACCT 5580
1801 V V F F L I T V L I Q Y R F F I R P R P 1820
5581 GTAAATGCAAAGCTATCTCTCTGAATGATGAAGATGAAGATGTGAGGCAGGAAAGACAG 5640
1821 V N A K L S P L N D E D E D V R R E R Q 1840
5641 AGAATTCTGATGGTGGAGGCCAGAAATGACATCTTAGAAATCAAGGAGTTGACGAAGATA 5700
1841 R I L D G G G Q N D I L E I K E L T K I 1860
5701 TATAGAAGGAAGCGGAAGCCTGCTGTTGACAGGATTGCGTGGCATTCTCCTGGTGAG 5760

1861 Y R R K R K P A V D R I C V G I P P G E 1880
 5761 TGCTTTGGGCTCCTGGGAGTTAATGGGGCTGGAAAATCATCAACTTCAAGATGTTAACAA 5820
 1881 C F G L L G V N G A G K S S T F K M L T 1900
 5821 CGAGATACCACTGTTACCAAGAGGAGATGCTTCCTAACAGAAATAGTATCTTATCAAAC 5880
 1901 G D T T V T R G D A F L N R N S I L S N 1920
 5881 ATCCATGAAGTACATCAGAACATGGCTACTGCCCTCAGTTGATGCCATCACAGAGCTG 5940
 1921 I H E V H Q N M G Y C P Q F D A I T E L 1940
 5941 TTGACTGGGAGAGAACACGTGGAGTTCTTGCCCTTTGAGAGGAGTCCCAGAGAAAGAA 6000
 1941 L T G R E H V E F F A L L R G V P E K E 1960
 6001 GTTGGCAAGGTTGGTGGAGTGGGCGATTGGAAACTGGGCTCGTGAAGTATGGAGAAAAA 6060
 1961 V G K V G E W A I R K L G L V K Y G E K 1980
 6061 TATGCTGGTAACTATACTGGAGGCAACAAACGCAAGCTCTACAGCCATGGCTTTGATC 6120
 1981 Y A G N Y S G G N K R K L S T A M A L I 2000
 6121 GGCGGGCCCTCTGTGGTGTCTGGATGAACCCACCACAGGCATGGATCCAAAGCCCCGG 6180
 2001 G G P P V V F L D E P T T G M D P K A R 2020
 6181 CGGTTCTGTGAAATTGTCCTTAAGTGTGTCAGGAGGGAGATCAGTAGTGCTTACA 6240
 2021 R F L W N C A L S V V K E G R S V V L T 2040
 6241 TCTCATAGTATGGAAGAATGTGAAGCTTTGCACTAGGATGCAATCATGGTCAATGGA 6300
 2041 S H S M E E C E A L C T R M A I M V N G 2060
 6301 ACGTTCAAGGTGCTTGGCAGTGTCCAGCATCTAAAAAATAGGTTGGAGATGGTTATACA 6360
 2061 R F R C L G S V Q H L K N R F G D G Y T 2080
 6361 ATAGTTGTACCAATAGCAGGGTCCAACCCGGACCTGAAGCCTGTCCAGGATTCTTGGA 6420
 2081 I V V R I A G S N P D L K P V Q D F F G 2100
 6421 CTTGCATTCTGGAAAGTGTCCAAAAGAGAACACCGAACATGCTACAATACAGCTT 6480
 2101 L A F P G S V P K E K H R N M L Q Y Q L 2120
 6481 CCATCTCATTATCTCTGGCCAGGATATTCAAGCATCCTCTCCAGAGCAAAAGCGA 6540
 2121 P S S L S S L A R I F S I L S Q S K K R 2140
 6541 CTCCACATAGAAGACTACTCTGTTCTCAGACACACTTGACCAAGTATTGTGAACCTT 6600
 2141 L H I E D Y S V S Q T T L D Q V F V N F 2160
 6601 GCCAAGGACCAAAAGTGTGATGACCAACTTAAAGACCTCTCATTACACAAAAACAGACA 6660
 2161 A K D Q S D D D H L K D L S L H K N Q T 2180
 6661 GTAGTGGACGTTGCAGTTCTCACATCTTCTACAGGATGAGAAAGTGAAGAAAGCTAT 6720
 2181 V V D V A V L T S F L Q D E K V K E S Y 2200
 6721 GTATGAAGAATCCTGTCATACGGGGTGGCTGAAAGTAAAGAGGGACTAGACTTCTT 6780
 2201 V *
 6781 GCACCATGTGAAGTGTGAGGGAGAAAGAGCCAGAACAGTTGATGTGGGAAGAAGTAAACTG 6840
 6841 GATACTGTACTGATACTATTCAATGCAATTCAATG 6880

Figure 3

5' 1 GTACCCCCCT TGCCTGGTTG ATCCTCAGGG TTCTACTTAG AATGCCTCGA

51 AAAGTCTTGG CTGGACACCC ATGCCAGTC TTTCTGCAGG GTCCCATTGG
101 GGTAAACCTT CTCATTCAT CCCATGTGAA CCAGGCCAGG CCCATCAGGG
151 TTTGGCAACC CCCTGATGCA GTGGTTGCTG CCAGGTGACA GGAGCAAGCC
201 TGCAGCTGCT GGGGGGCCAT GCAGAGACAG CCTGCCAGAG GGGAGACCAC
251 CTGGGGAGGC CAGAGCCGTG GAGACAGCAA GAGACCAGGG GCTGAGGACA
301 GAGTAGTACA GGTCTTTGGT CCCAGTAGTC CTGAAACCCAC TGCACTCCGA
351 ACCTTCTGT ACTTAGCTTA AGCCAGTTGG AGTTTCTGTC CTTTACAACC
401 AAGAGCCTG ATAGGAATGG GGTCCTGTGC TACGCTACTG TTGGCTTCTT
451 TCCCGATCGG GCGCTGGAGG GGAACACAGC AGTGACTACA GTGGGATGCT
501 TACTCGGTGC TGGGCATGCT AGAAAAGTGC TGCCATGCCT TATTTCCAC
551 GTGGTGGGGA TTTTGACCCC ACCTGTACAG ACAGATAAGT GAGGACCCCTT
601 TTCACCTTAT CCTGCAACAG AAAATCCAGC AGCCAAAGCC AACAAAGGGCC
651 CAGCATAGCA TCTTCCCTCT CTGACTTCAT CCTCACGCTC CACACACCAT
701 CCCCCCTGGCC ATTCCCAGCA GCCCAGTAAG CACTGCCCTCA CACTTCCAGT
751 TCCGGACCAG CCAGGATGCC CAGGCTGGAT GGGGGCCATC CACCGGCTGA
801 AGCCAATTGC CTATTCTCGA GCTGAAGGTG AATCAATCCC GCATAAAATCT
851 TCAGGGCAGAG AACTNGGGTG GGGGGTAGAA GAGGGGAAT GTCTAGAAGG
901 AAATTCTGGG GCACATTCTC GGAAGTGAGG AGGATGGATA TTGGACAGAA
951 ATTATGTCA TGCAGGCACC CTCACTTGCC CTGGCCACAT GGACAGTTCC
1001 TCCCCGGCTG TGTCCCGNGC CTCCCTCTCGT GCTCCAGGGC CTGCTCTGTT
1051 CTGGAGCGAG ATGGGTCCCA GGGCTGGCA CCAGTCCCCA TCTCCAGGCCA
1101 TCAGGCACCTT TCCTCTCTGT GTTTTGGCGT AAACACNTCC CTAGGTTTGT
1151 GGATCTGAAT CCTCTTCCCA ACACACTCAA GCTTTGCTGG GCCTCCCTGC
1201 AGTGTATGTT TAAGGCACCA CACAGCCTCC AAGGCCCTGC ACCCGGGCAG
1251 TGGCCACCTG GTAAACACAG CAGTCAGATT TCCTCATTTC AGCCAAGTGT
1301 AAAATCAAGG TAATGGATCT ACNCTTTTT TTTTNNTNTT TTCCAGGGG
1351 GNTNNNTTTT TTTGAGACG GAGTCTCACT CTGTCANCCC CGGTCTGGAG
1401 TGCAGTGGCT CAATCTCGGC TCANCTGGCA AGCTCCGCCT CCCAGGTTCA
1451 TGCCATTCTC CTGCCTCAGC CTACATAGTA GCTGGGACTA CAGGTGCCCG
1501 CCACCCACACC TAGCTAATT TTTGTATTT TAGTAGAGAC GGGGTTTCAT
1551 CATGTTAGCC AGGATGGTCT CGATCTCCTG ACCTCCAAA GTGGTGGAG
1601 TTACAGGTGT GAGCCACTGC GCNCCGGCTG GATGACTCTT GAGACAACAC
1651 CATTCAAGACA AAGGCAAGGC CTCCCACCTTA AACTCATAAAC CGTGTCTCCT
1701 TTCTCTCCTT CGATTGAGC GGCTGAATT GGTTACAGTC ATCTGACCTG
1751 TGGGTGTGAA NGTCCACCTG CCTGGCATAA AAAGCTGTGC CTCTTTCTA
1801 GGTGAGGAGA AAGAGAGAGA CCTGGCTCAT CTGAGGTGTG GTTGGGAGGG
1851 GGGACCCAGG TGTGCTGGAA ATGAAAAGAA ATGCATTCCCT GTTTTTTCGT
1901 CCCAACATGC AAACAACCTGA ACAAAAGCAT TAGGGCCTGA GACTGGGAGT
1951 AAAGAATTCC TTGTCACCAT GGATACCAGG AAATGCCCA ACTTATATAT
2001 AATAAGGGCT TTAGAGATGC TGGACCACCT GATATTCCAG CCTGGGGCCA
2051 CATGGGAGTG TGCCCTGGTG TTATTCCTTA TACAGTTCCA TGAACATGGC
2101 TCTGGAAACA CCTCTGTCTG CAGAAAATGA GGCTTTCTT TTTTGTTCG

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2151 GGGGTGAACA GAGGGCAGAG GCCTGGCAT CTTCACTCAG CACCCCTTTG
 2201 TAACCCAGCA CTTAGCACCA TGGCTGGCGC ACAGCAATGT CACATGTGTG
 2251 AGTGCACACG ATGCCCACT GCCAGGGTC ACCCCACACC GGTGCTGTTG
 2301 GGGGCGTTGG AGTGGTTATC TCTTCTTAG TCCTCAAGCT CCTACCTGGC
 2351 AGAGAGCTGC CCAACACCGT CGGGGTGGGG TGGGCGGGAA GGGAAAGAAGC
 2401 AGCAGCAAGA AAGAACCCCC CTGGCCCTCA CTCTCCCTCC CTGGACGCC
 2451 CCTCTTCGAC CCCATCACAC AGCCGCTGA GCCTGGAGN CAGTGGATT
 2501 CCGAGCCTGG GAACCCCCGG CGTCTGTCCC GGTGTCCCCC GCAGCCTCAC
 2551 CCNCGTGCTG GCCCCAGCCCC CGCGAGTTCG GGACCCGGGG TTCCCGGGGT
 2601 GCGAGGGGGT TCCCATGCCG CCTGCGAGGC CTGCGCTCGG GCGCTCCCG
 2651 GAAACCTGCAC TTCAGGGTC CTGGTCCGCC GCCCCCAGCA GGAGCAAAC
 2701 AAGAGCACGC GCACCTGCCG GCCCAGCCGC CCCCTGGTG CCGGCAATC
 2751 GCGCGCTCGG GGCGGGGTG GCGCGCTGG AACCAAGAGCC GGAGCCGGAT
 2801 CCCAGCCGGA GCCAAGCGC AGCCCGCACC CCGCGCAGCG GCTGAGCCGG
 2851 GAGCCAGCGC AGCCTCGGCC CCGCAGCTA AGCCTCGTCC CCGCCGCCNG
 2901 CCGCCGCACG CCGCCGCCGC CGCCCCCGGG GCATGGCTGT CTGATGGCCG

EXON1/INTRON 1

2951 CTTTCTCGGT CGGCACCGCC ATGGTGAGTG AGCGCATCCT TCGTCCGCCG
 3001 GGAACGGTTT TATTTCAAG GAGAGCAGGA AACACACAAA GACTCGCAAG
 3051 CTCGACCTGA CACCCCTCCC AGGAGCGCGT CCTCTGGGGC GCTGACCCAG
 3101 GGGCACCCCTA GAGTGGCGCC CGGCTCCGAT CGCTGCCCT NNCCCTCCG
 3151 CCAGGGCCAC CTGGGAGCCG CGGGGATGCC CCTTGACCCG GCAGAGNGCA
 3201 CGGACTAGGT GGAGGGGNCC GGGATTGGGG CGGGGGGCAG NCAGTTGCC
 3251 TACAAGTTGG ACCGATGGCC TTGACCTGAT GGCTTCTGGG CGGGGGCGT
 3301 GGGGAGCTGG GGACCCGGAG CGCACTGGGG ACTGGGGAGG GCGCGCAGCT
 3351 TGGGCCGGAG GGAAGAGGGG ACTTGAAGAA GGGGAGCCCC GCGCGCGCG
 3401 CTGTGGGCTT GGGGACCCGG GACTTCTCGC GCCATCCCCA GGAACGCCAG
 3451 GCAAGGTCTG GGGAACAAAA GAGGAAGCTG CCCCCAGAGA GCCGGAGCTC
 3501 GACTGNACTC CC 3'

Figure 4

5'

1 CTTGGTGGCG CATGCATCGT GGTGCTCATC TTTCTGGCCT TCCAGCAGAG
 51 GGCATATGTG GCCCCCTGCCA ACCTGCCTGC TCTCCTGCTG TTGCTACTAC
 101 TGTATGGCTG GTCGATCACA CGCCTCATGT ACCCAGCCTC CTTCTCTTC
 151 TCCGTGCCCA GCACAGCCTA TGTTGGCTC ACCTGCATAA ACCTCTTTAT
 201 TGGCATCAAT GGAAGCATGG CCACCTTGTG GCTTGAGCTC TTCTCTGATC
 251 AGAAGCTGCA GGAGGTGAGC CGGATCTGA AACAGGTCTT CCTTATCTC
 301 CCCACTCTG CTTGGGCCGG GGGCTTATTG ACATGGTGC GNAACCAGGC
 351 CATGGCTGAT GCCTTGTGANC CCTTGGGAAA AAGGCAGTTC AAGTACCCCTG

401 NCTTGGAAAGG TGGCGGAAGA ACCTTTGGC ATGGGAACAG GGCCCCCTTT
451 CCTTCTCTTC ACACTANTGT TCAAGCACCG AAGCCAACTC NTGCCACAAG
501 CCCAGGTAAG GTCTCTGCCA CTCCCTGGAGA GAGACGAGGA TGTAGCCCCT
551 GAACGGGAGC GGGTGGTCCA AGGAGGCCACC CAGGGGGATG TGTTGGTGCT
601 GAGGAACCTG ACCAAGGTAT ACCGTGGGCA GAGGATGCCA GCTGTTGACC
651 GCTTGTGCCT GGGGATTCCC CCTGGTGAGT GTTTTGGGCT GCTGGGTGTG
701 AACGGAGCAG GGAAGACGTC CACGTTTCGC ATGGTGACGG GGGACACATT
751 GGCCAGCAGG GGCGAGGCTG TGCTGGCAGG CCACAGCGGG CCCGGGAACC
801 CAGTGTGCAGC ACCTCNAGGG CAGGCNCAGC GTGGCCCGGG AACCCAGTGC
851 TGCGCACCTA AGCATGGGAT ACTGCCCTNA ATCCGATGCC ATCTTGAGC
901 TGCTGACGGG CGCGGAGCAC CTGGAGCTGC TTGCGCGCCT GCGCGGTGTC
951 CCGGAGGCC AGGTTGCCA NACCGNTGGC TCAGGGCCTGG CGCGTCTGGG
1001 ACTCTCATGG TACGCAGACC GGCGTGCAGG CACCTACAGG AACCTGCCCG
1051 GGCGGCCGCT CGAGCCNTA NNTGAAGTA 3'

Figure 4b

...CTCCTGCCAC AGTTAGTGAG GTCTATGGAG AGGGTGGCAG GGGCCAAAGGA
CCTACTTTAA GCCCACAGAT ATTCTGTCCC CAGGCCAGG GTGAGGTCTC...

Figure 5

CDNA-sequences of lipid sensitive Genes:

*ABCB9, ABCA6, ABCC4, ABCA1, ABCD2, ABCB1, ABCB4, ABCC2, ABCD1, ABCC1,
ABCB6, ABCB11, ABCG2, ABCC5, ABCA5, ABCG1, ABCA3*

ABCB9 GENBANK:U66676

GCCAATGNCACGGTTCATCATGGAACTCCAGGACGGCTACAGCACAGAGACAGGGGAGA
 AGGGCGCCCAAGCTGTCAAGGTGGCCAGAACCCAGCGGGTGGCCATGGCCNGGCTCTGGTGC
 GGAACCCCCCAGTCCTCATCTGGATGAAGGCCACCAGCGCTTTGGATGCCAGAGCGAGT
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ABCG1 Acc.Nr.: U34919 GENBANK: HSU34919

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Fragment 698739

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Ser Gly Met Asp Gly Lys Gly Ser Tyr Gln Val Lys Gly Trp Lys Leu
1250 1255 1260

Thr Gln Gln Gln Phe Val Ala Leu Leu Trp Lys Arg Leu Leu Ile Ala
1265 1270 1275 1280

Arg Arg Ser Arg Lys Gly Phe Phe Ala Gln Ile Val Leu Pro Ala Val
1285 1290 1295

Phe Val Cys Ile Ala Leu Val Phe Ser Leu Ile Val Pro Pro Phe Gly
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Lys Tyr Pro Ser Leu Glu Leu Gln Pro Trp Met Tyr Asn Glu Gln Tyr
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Thr Phe Val Ser Asn Asp Ala Pro Glu Asp Thr Gly Thr Leu Glu Leu
1330 1335 1340

Leu Asn Ala Leu Thr Lys Asp Pro Gly Phe Gly Thr Arg Cys Met Glu
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Gly Asn Pro Ile Pro Asp Thr Pro Cys Gln Ala Gly Glu Glu Glu Trp
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Thr Thr Ala Pro Val Pro Gln Thr Ile Met Asp Leu Phe Gln Asn Gly
1380 1385 1390

Asn Trp Thr Met Gln Asn Pro Ser Pro Ala Cys Gln Cys Ser Ser Asp
1395 1400 1405

Lys Ile Lys Lys Met Leu Pro Val Cys Pro Pro Gly Ala Gly Gly Leu
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Thr Gly Arg Asn Ile Ser Asp Tyr Leu Val Lys Thr Tyr Val Gln Ile
1445 1450 1455

Ile Ala Lys Ser Leu Lys Asn Lys Ile Trp Val Asn Glu Phe Arg Tyr
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Gly Gly Phe Ser Leu Gly Val Ser Asn Thr Gln Ala Leu Pro Pro Ser
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Gln Glu Val Asn Asp Ala Thr Lys Gln Met Lys Lys His Leu Lys Leu
1490 1495 1500

Ala Lys Asp Ser Ser Ala Asp Arg Phe Leu Asn Ser Leu Gly Arg Phe
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Met Thr Gly Leu Asp Thr Arg Asn Asn Val Lys Val Trp Phe Asn Asn
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Lys Gly Trp His Ala Ile Ser Ser Phe Leu Asn Val Ile Asn Asn Ala

1540

1545

1550

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Ile Thr Ala Phe Asn His Pro Leu Asn Leu Thr Lys Gln Gln Leu Ser
1570 1575 1580

Glu Val Ala Pro Met Thr Thr Ser Val Asp Val Leu Val Ser Ile Cys
1585 1590 1595 1600

Val Ile Phe Ala Met Ser Phe Val Pro Ala Ser Phe Val Val Phe Leu
1605 1610 1615

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1620 1625 1630

Val Lys Pro Val Ile Tyr Trp Leu Ser Asn Phe Val Trp Asp Met Cys
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Asn Tyr Val Val Pro Ala Thr Leu Val Ile Ile Phe Ile Cys Phe
1650 1655 1660

Gln Gln Lys Ser Tyr Val Ser Ser Thr Asn Leu Pro Val Leu Ala Leu
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Leu Leu Leu Leu Tyr Trp Ser Ile Thr Pro Leu Met Tyr Pro Ala
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Ser Phe Val Phe Lys Ile Pro Ser Thr Ala Tyr Val Val Leu Thr Ser
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1715 1720 1725

Glu Leu Phe Thr Asp Asn Lys Leu Asn Asn Ile Asn Asp Ile Leu Lys
1730 1735 1740

Ser Val Phe Leu Ile Phe Pro His Phe Cys Leu Gly Arg Gly Leu Ile
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Asp Met Val Lys Asn Gln Ala Met Ala Asp Ala Leu Glu Arg Phe Gly
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Glu Asn Arg Phe Val Ser Pro Leu Ser Trp Asp Leu Val Gly Arg Asn
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Leu Phe Ala Met Ala Val Glu Gly Val Val Phe Phe Leu Ile Thr Val
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Leu Ile Gln Tyr Arg Phe Phe Ile Arg Pro Arg Pro Val Asn Ala Lys
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Leu Ser Pro Leu Asn Asp Glu Asp Val Arg Arg Glu Arg Gln
1825 1830 1835 1840

Arg Ile Leu Asp Gly Gly Gln Asn Asp Ile Leu Glu Ile Lys Glu
1845 1850 1855

Leu Thr Lys Ile Tyr Arg Arg Lys Arg Lys Pro Ala Val Asp Arg Ile
1860 1865 1870

Cys Val Gly Ile Pro Pro Gly Glu Cys Phe Gly Leu Leu Gly Val Asn
1875 1880 1885

Gly Ala Gly Lys Ser Ser Thr Phe Lys Met Leu Thr Gly Asp Thr Thr
1890 1895 1900

Val Thr Arg Gly Asp Ala Phe Leu Asn Arg Asn Ser Ile Leu Ser Asn
1905 1910 1915 1920

Ile His Glu Val His Gln Asn Met Gly Tyr Cys Pro Gln Phe Asp Ala
1925 1930 1935

Ile Thr Glu Leu Leu Thr Gly Arg Glu His Val Glu Phe Phe Ala Leu
1940 1945 1950

Leu Arg Gly Val Pro Glu Lys Glu Val Gly Lys Val Gly Glu Trp Ala
1955 1960 1965

Ile Arg Lys Leu Gly Leu Val Lys Tyr Gly Glu Lys Tyr Ala Gly Asn
1970 1975 1980

Tyr Ser Gly Gly Asn Lys Arg Lys Leu Ser Thr Ala Met Ala Leu Ile
1985 1990 1995 2000

Gly Gly Pro Pro Val Val Phe Leu Asp Glu Pro Thr Thr Gly Met Asp
2005 2010 2015

Pro Lys Ala Arg Arg Phe Leu Trp Asn Cys Ala Leu Ser Val Val Lys
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Glu Gly Arg Ser Val Val Leu Thr Ser His Ser Met Glu Glu Cys Glu
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Leu Gly Ser Val Gln His Leu Lys Asn Arg Phe Gly Asp Gly Tyr Thr
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2130

2135

2140

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Leu Asp Arg Glu Asp Leu His Cys Asp Ile Asp Glu Thr Cys His Phe
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Gln Lys Ser Glu Ala Ile Leu Arg Glu Leu Asp Val Glu Asn Ala Lys
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Leu

65

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<213> Human

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<213> Human

<220>

<223> human Intron-Sequence of ABCA8 (ABC-new)

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<210> 22

<211> 15

<212> DNA

<213> Human

<400> 22

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<210> 23

<211> 372

<212> DNA

<213> Human

<220>

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<400> 23

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<211> 281

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<213> Human

<220>

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<400> 24

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<210> 25

<211> 2258

<212> DNA

<213> Human

<220>

<223> human cDNA of Huwhite2

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<211> 820

<212> DNA

<213> Human

<220>

<223> human cDNA

<400> 26

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<211> 575

<212> DNA

<213> Human

<220>

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<400> 27

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<211> 300

<212> DNA

<213> Human

<220>

<223> human cDNA

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<212> DNA

<213> Human

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<220>

<223> human cDNA of ABCG2

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2719

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<212> DNA

<213> Human

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<223> human cDNA of ABCA3 (ABC3)

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